# INFLUENCES OF LOW TEMPERATURE ON COLD HARDINESS AND NEURAL ADAPTATION IN THE ALASKAN BEETLE *PTEROSTICHUS BREVICORNIS* (CARABIDAE)

A

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INFLUENCES OF LOW TEMPERATURE ON COLD HARDINESS
AND NEURAL ADAPTATION IN THE ALASKAN BEETLE
PTEROSTICHUS BREVICORNIS (CARABIDAE)

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## ABSTRACT

A species of adult carabid beetle, Pterostichus brevicornis, has been found to overwinter in the Fairbanks locale within decayed stumps. Empirical observations indicated that body freezing was tolerated. Indications of continued vital processes throughout the overwintering period were also noted. In light of these observations the existance of a cryoprotective compound was suspected and determined to be glycerol.

The hemolymph glycerol of adult Pterostichus brevicornis was determined utilizing quantitative paper chromatography. Seasonal variations were revealed in the concentration of this substance. Mean glycerol levels in excess of 22 gm% have been measured during winter with values decreasing to less than 1 gm% during spring and summer. Acclimation studies gave similar results. In the artificially warmed winter beetles glycerol content decreased to less than 1 gm% within 36 hours at 20°C. The stimulus to the iniation of glycerol synthesis was found to be first exposure to 0°C following summer. The rate of accumulation varied nearly directly with sub-zero (°C) exposure temperature.

Glycerol concentrations were closely correlated with the changing whole body supercooling and hemolymph freezing points in both naturally acclimatized and laboratory acclimated specimens. Both supercooling and freezing points were depressed 0.9°C per 4 gm% increased in glycerol. Hemolymph glucose and trehalose levels were also correlated

with fluctuations in glycerol in an attempt to define the probable source of glycerol. Glucose did not vary seasonally whereas trehalose changed significantly.

Temperature fluctuations within the hibernacula were considered. Also, seasonal variations in locomotor response to temperature were determined in a thermal gradient chamber and correlated with changes in cold tolerance. Mean temperature preferences were found to vary from a summer high of +13.3°C to a winter low of -5.5°C. Sub-freezing exposures were avoided unless glycerol was present.

Attempts were made to integrate the above observations with neural function. Electrical recordings from the region of the modified trochanter on the hind thoracic legs revealed a thermosensitive region. Semi-microelectrode recordings have evidenced motor fiber populations displaying narrow bands of differential temperature sensitivity. Activity in some fibers has been recorded at temperatures as low as -11.7°C. These fibers vary their tonic discharge patterns with varying states of acclimation and acclimatization, thereby allowing continued activity at sub-zero temperatures. Localization of discharge to motor efferents was made utilizing neurophysiological, surgical and neurohumoral techniques. Fluctuations in the discharge frequencies are thought to reflect changes in nerve tone.

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# INTRODUCTION

## History of Insect Freezing Survival

The earliest records of observations relating to the ability of insects to survive freezing are found in Aristotle's History of Animals (translated by Cresswell, 1902). Casual recollections of a larva's ability to withstand freezing were noted but without further comment. Such lack of interest was due to the philosophical misgivings of this early period coupled with the absence of quantitative natural sciences. A gap of nearly two thousand years followed in which scientific investigations related to low temperature were either non-existent or obscure.

The scientific impetus realized during interim periods led Robert Boyle (1683) to conduct experiments on the physical, chemical and biological effects of cold. His observations on cryopreservation of fruits, meats, eggs and other perishables are considered classic. Boyle also experimented with whole body freezing of frogs and small fish to discover that they survived in the presence of an external solid ice medium.

It was not until the advent of the thermometer that direct measurement and reproducibility could be obtained regarding thermal phenomena. In 1730 Reaumur modified Fahrenheit's original thermometer of 1714 by replacing the alcohol column with mercury. These developments represented early landmarks for low temperature science.

Reaumur (1736) was among the first to experiment with the freezing of insects. During the course of experimentation, he made a number of

observations basic to cryosurvival and which were not appreciated or understood until nearly two hundred years later. Caterpillars were cooled for 30 minutes at -19°R (-22.5°C) and upon controlled, slow rewarming, survived. A second species was found not to survive freezing at -15°R (-17.5°C). The latter group did not survive any internal freezing but tolerated cooling to -9°R (-11°C). One might consider this the earliest record of supercooling within insects although a specific term was not applied. No caterpillar was found to survive complete body freezing, and some liquid must have remained for life according to Reaumur's concepts.

Observing the differences in survival between insects, Reaumur compared each species with strong or weak brandies with respect to their "freezing" points. Further experiments demonstrated differences between insects naturally exposed to winter cold and those sheltered beneath the ground. He stated that,

> "...the insects which remain exposed to the greatest cold are most able to withstand it. Those which are more sensitive to cold act as if they can foresee what will happen during winter on the surface of the earth and which they will not be able to withstand. I say that they can foresee it because it is not the approach of winter or the actual cold which determines the time at which they bury themselves in the ground. We have seen that some caterpillars bury themselves in July and August and still others that do this in the beginning of spring."

One may summarize Reaumur's early contributions by stating the concepts contained within his studies. The significance of cooling rates, time at a given low temperature and rewarming rates were noted. Supercooling was alluded to, but the concept was beyond the scope of knowledge available in the 1730's. Freezing point variations of individual species were thought due to chemical differences in the blood. Finally, it was realized that habitat temperature need not be the primary stimulus to diapause or hibernation.

In the course of the following 150 years new insights were gained into the understanding of insect freezing survival. Numerous workers studied the periodicity of hibernation (Huber, 1792; Kirby and Spence, 1818; Vaudoner, 1827 and Scudder, 1889) as related to feeding, reproduction and temperature while others concerned themselves with measurements of body temperature (Newport, 1837 and Bachmetjew, 1901). Bachmetjew provided the most comprehensive study utilizing the thermocouple to determine freezing points and body temperature. While much of his work was later criticized, he made three important observations. First, the time rate of cooling affected the supercooling points; second, the percent of "sap" (water) was found to be inversely proportional to the freezing point; and, third, lethality occurred when the supercooling point was reached for the second time. His shortcomings were found in his attempts to extrapolate to all insects from the particular ones studied.

Further correlations between low temperature survival and season were obtained by Duval and Portier (1921) and Knight (1922). Reliability

was enhanced as a result of the work of Carter (1925). He found that injury (piercing an insect with a thermocouple) affected not only survival but supercooling points. Simultaneously, a number of workers emphasized the probable role of dehydration in freezing survival. However the solution to the problem of survival remained obscure.

Investigators soon became aware of the differences not only between individuals in different states of development, reproduction and feeding within a given species but also between species. Theories of cold resistance that offered generalized mechanisms for the entirety of the insect kingdom (Bachmetjew, 1901) were soon cast aside. The principles established by Reaumur were reaffirmed while later incorrect concepts were discarded. Much of this refutation came in the form of works later to be deemed questionable (Sacharov, 1930).

Payne (1926, 1927a and b) did much to clarify the situation left unresolved by earlier workers. Her extensive and thorough studies on insect cold resistance were well organized. Supercooling and freezing points of various insects exposed to varying environmental conditions were determined seasonally. Both supercooling and freezing points were found to oscillate periodically being lowest in winter and highest in summer. Insects resident in stored products or aquatic situations were not found to vary these parameters seasonally. By applying artificial thermal stimuli, Payne demonstrated that cold hardiness was dependent on temperature by raising and lowering supercooling and freezing points in response to varying acclimation temperature. It should be noted that the phenotypic expression of cold resistance could only be induced in

those insects naturally demonstrating this adaptation. Finally, Payne (1927a) found that cold resistance may be facilitated by either dehydration or starvation. The former results in a more rapid survival response.

Further physico-ecological studies continued. Ludwig's (1928) studies on Japanese beetles showed that variations during the individual's development were important in determining the response to low temperature. The larval stage normally found to overwinter responded most favorably to cold. Uvarov (1931) provided an excellent and comprehensive review of knowledge concerning insects and climate in general. While not directly working in the field of insect freezing survival, Uvarov attempted to integrate the works of Payne, Sacharov, Bachmetjew and others to formulate a theory of low temperature survival. However, realizing the complexity of the situation he could only conclude that it "... is obvious that the problem is intimately connected with the most obscure phenomena of the bio-chemistry and biophysics of metabolic water in living organisms, and it has to be attacked with corresponding methods instead of crude qualitative experiments".

The period between Uvarov's monograph and the early 1950's represented a scientific calm in many areas of research. The few studies undertaken during this period were primarily concerned with improvements of techniques (Ditman, et al., 1942 and 1943).

Scholander, Flagg, Hock and Irving (1953) provided the first revival of interest in this field. Their study of the Alaskan midge, Chironomus, which overwinters in ice or frozen mud at the bottom of

arctic pools, provided a fresh approach in light of the development and utilization of new procedures. These investigators demonstrated survival of the midge at temperatures as low as -40°C. At -15°C unfrozen water was estimated to be only 10% and at -35°C little free water remained. Oxygen consumption was found to decrease throughout the period of reduced temperature with a precipitous drop evident between 0° and -15°C. $Q_{10}$ values increased from 4 to 50 between 0° and -5°C. This result indicated that the survival advantage of an organism that tolerates freezing is amplified by the tremendous reduction in energy requirements. Also, upon warming (thawing) to 0°C a boost in metabolism by a factor of twelve would be evident. Based upon the observation that survival time was inversely related to metabolic rate, Scholander, et al. (1953) theorized that "at a $Q_{10}$ of 50 an organism capable of surviving a modest 10 days at 0°C would last for a thousand years at -23° and for a million years at -42°C!" They did, however, indicate that the basis for this statement was a mathematical extrapolation and of questionable biological significance. Evidence will be presented in chapters 1 and 2 which tends not to substantiate this statement, at least in Pterostichus brevicornis.

In 1954 Asahina, Aoki and Shinozaki reported that the prepupae of Monema survived freezing to -30°C following supercooling to -20°C in the overwintering form. The summer larvae did not survive freezing at -10°C. Microscopic studies by these same workers showed that freezing was extracellular in the overwintering stage while intracellular in the summer form. In a further study Asahina and Aoki (1958) demonstrated

within this same pupa that survival resulted after exposure to -90°C for 45 minutes providing cooling rates were approximately 1°C per minute. Also, temperatures as low as -180°C were tolerable if the pupa was slowly precooled to -30°C and then rapidly cooled.

These studies, while interesting, shed little light on the mechanisms involved in survival. The question as to how one insect survived a given freezing temperature and why a second did not remained unanswered. In 1957 Wyatt and Kalf found that glycerol was a major component of the hemolymph of the pupa Hyalophora cecropia. Independently, Chino (1957) made a similar observation in the diapausing eggs of the silkworm Bombyx while Salt (1957) found glycerol in the gall fly larvae, Eurosta solidaginis, and the webworm, Loxostege sticticalis. In all cases, glycerol was found in an immature, overwintering form. The direct significance of these observations was somewhat obscured by apparently conflicting results. Salt (1957) noted that both Eurosta and Loxostege contained approximately 2-4% glycerol but that Loxostege was not freezing tolerant. In 1959 Salt reported that the larvae of Bracon cephi contained glycerol concentrations as high as 25% while overwintering. Freezing points were depressed to as low as -17.5°C while supercooling points were lowered to -47°C. The source of glycerol was considered and found not to be glycogen and probably not lipid (?). One other significant observation made in the course of this study was that glycerol accumulation and loss was seasonally periodic. Glycerol obviously acted in this insect to enhance cold hardiness by decreasing the supercooling point to avoid freezing and to protect if freezing occurred. The significance of

large concentrations being necessary to afford sufficient protection in certain insects was realized. Other workers offered evidence supporting the hypothesis that glycerol was protective. Wilbur and Mahan (1958) demonstrated enhanced viability of heart tissue from the beetle Popilius. Dubach, et al. (1959) measured glycerol in the eggs and adults of carpenter ants at levels of 10% in a northern Minnesota population while a Maryland group contained no glycerol during the same seasonal period.

Salt (1959) made an important observation relating to intracellular freezing. In the cold hardened larva of Eurosta, cells of the fat body survived freezing. Size, shape and organelle arrangement were not changed during freezing and rewarming. The only observable change noted was the coalescence of oil droplets. Losina-Losinsky (1967) conducted similar studies and found that tracheal and salivary gland cells of the corn borer, Pyrousta, survived intracellular ice at temperatures below -200°C. Cooling rate was moderate while rewarming was rapid. The results of both Salt (1959 and 1962) and Losina-Losinsky (1967) have been questioned on theoretical bases by Asahina (1966) but not experimentally disproven. Further analyses are required.

Two excellent reviews are available discussing the foregoing studies (Smith, 1961 and Salt, 1961). The review by Salt is of particular interest for it contains many of the concepts basic to the experiments presented in this dissertation.

Salt (1961) distinguished three principles of cold hardening. These were: (1) cold acclimation, (2) avoidance of freezing by

supercooling, and (3) freezing tolerance.

Cold acclimation (and acclimatization) studies have dealt with temperature dependent changes in rates of reaction (metabolism, activity, stimuli reactions, etc.) and have ignored those activities not considered "necessary" for survival (growth, reproduction, etc.). Bullock (1955) advocated this concept in his temperature compensation approach to poikilotherms. Generally, a cold adapted insect maintains a higher level of activity at a given temperature than a warm adapted form. Such cold acclimation represents a potential but not a direct advantage.

Freezing avoidance by supercooling is probably the most common method of cold hardening employed by insects. All insects supercool at least to a limited degree, the extent of which may usually be correlated with exposure temperatures and adaptations possessed. Supercooling also occurs in freezing tolerant forms. It is not necessarily dangerous to an insect. Metabolically, it represents only an extension of activity to below freezing temperatures (Scholander, et al., 1953; Bullock, 1955 and Salt 1958), provided chill coma does not occur. For many insects, the supercooling point represents the lower lethal limit. A discussion of the influence of glycerol on depression of supercooling points may be found in Chapter 2.

It becomes apparent that the real concern is with freezing at the termination of supercooling and therefore the phenomena associated with freezing. This necessitates a brief review of nucleation and ice formation. It is known that as temperature decreases, the

molecular motion diminishes and individual molecules assume a position closer to each other increasing the probability for chance orientation into an ice lattice. These molecular aggregations are thought to shrink and grow in response to temperature fluctuations. When an aggregation attains a critical size (the nucleus), freezing occurs and ice growth proceeds via accretion. The rate of growth is fast enough to advance the ice front throughout the insect's body in a fraction of a second while precluding the formation of additional nuclei. Crystal growth is slowed in proportion to increasing viscosity, and in a very viscous system, many nuclei may form. It should be noted that conditions favoring nucleation must exist or supercooling will persist.

This discussion has assumed that nucleation was homogeneous (only water contributed to the nucleus formation). In insects and other tissue systems nucleation occurs by the addition of water to non-aqueous surfaces. Such nucleation is termed heterogeneous. The closer the resemblance of the surface to ice, the more efficient the nucleator will be. According to Salt (1961), nucleation is dependent on at least six factors: (1) number of efficient nucleators, (2) size of water mass, (3) time, (4) temperature (different substances nucleate at different temperatures), (5) position of nucleators (surface or within media), and (6) influence of surface forces. It was further pointed out that in a biological system the situation is simplified by the lack of control over indigeneous nucleating agents, aspects of surface and interface, and size and shape. The only readily controlable factors

are those of cooling rate and condition of the insect, i.e. nutritional or reproductive state, age, etc. Concern over the complex mixture of nucleating agents is unnecessary since the most efficient agent will cause the earliest freezing and thereby set the supercooling point.

To the insect within its winter habitat, nucleation may result from one of three processes: (1) nucleation in the digestive tract due to food and contaminants, (2) innoculation through the cuticle, or (3) time.

The digestive tracts of most cold hardy insects are generally evacuated. Feeding insects tend to be less cold hardy than non-feeding stages. One explanation of this observation is that food particles probably contain atmospheric nucleators (mineral particles) which are highly efficient nucleating agents. If the efficiencies of these particles are greater than those indigeneous to the insect, supercooling range will be decreased. Pterostichus brevicornis does not evacuate its gut prior to hibernating (Kaufmann, 1970) and doubtless contains an abundance of nucleators.

Innoculation may occur through the cuticle if ice forms and penetrates the cuticle. This appears unlikely due to the water-proof wax layer surrounding the cuticle. However, this layer is not perfect and is subject to damage thereby increasing the chance of nucleation if water condenses and freezes on the cuticle. Both these factors, food and contact moisture, can reduce supercooling but are usually not considered of great importance. This presumption was made by numerous workers due to the fact that immature stages were studied almost

exclusively. Larvae and pupae generally evacuate the gut during various developmental stages with or without a hibernating state (diapause). Those forms found in northern latitudes requiring lengthy development interrupted by winter would behave in this manner and not necessarily represent a special adaptation. Adult, overwintering insects have not been found by most earlier workers, and therefore were presumed not to exist. Asahina (1966) fosters this mis-conception by stating that in "... adult insects bodily freezing always results in fatal injury, even at high subzero temperatures, although some of them are very frequently found to be apparently normal just after thawing. This presumably reflects the relative inability of well differentiated tissues in an adult insect to resist frost injury." In the Fairbanks region more than two dozen species of insects have been found in the adult stage during mid-winter at temperatures as low as -40°C and have recovered upon rewarming. Many orders have been represented: Coleoptera, Hymenoptera, Lepidoptera, Homoptera, Diptera, etc., along with a number of Arachnids. It is probable that numerous other adult species exist for the search has been limited to decayed stump habitats. Kaufmann (unpublished) has also found numerous diverse adult forms ($\sim$ 30 species) in the same region.

The time required for the actual termination of supercooling and freezing is variable because nucleation depends on the probability of a particular molecular arrangement. Salt (1966a and b) pointed out the importance of time with respect to supercooling. If insects were

cooled at rates of a few tenths to a few degrees per minute, little difference in the supercooling points was noted. For example, cooling rates increased over 2000 times with only a 2°C decrease in supercooling points. However, if cooling extended over hours or days, freezing would likely occur at a higher temperature due to longer exposure below the normal freezing point (Salt, 1966). Hibernating insects spend long periods supercooled and their chance of freezing with time increases. For example, an insect cooled to -20°C freezes; if cooled only to -19°C, freezing may still occur but takes one minute; at -17°C, an hour; at -15°C, a day and at -10°C, a month (Salt, 1961). This assumes that the true freezing point is above -10°C. Supercooling can be extended by inhibiting nucleation in mixed aqueous systems via increased viscosity at lower temperatures and further augmented by increased solute levels (glycerol). Baust and Miller (1970) have confirmed this observation in P. brevicornis.

Some considerations of the influence of dehydration on cold-hardiness is warranted. Dehydration tends to concentrate solutes thereby lowering the freezing point and also the supercooling point. This would confer a limited but negligible degree of cold hardening. Salt (1956) has shown that cold hardiness was increased with drying but only to a slight degree until lethal levels were reached. Dehydration beyond lethal levels increased hardening but was of little value to the already dead insect. No cases are known whereby insects prepare for hibernation by dehydrating to the extent that their freezing points were lowered by several degrees.

Water content was observed to decrease in all hibernating insects, but this was due to the consequent reduction in the need for water by various tissues due to gut-evacuation, cessation or decrease of various secretory activities and accumulation of non-aqueous storage materials. Salt (1956) further found that even moderate dehydration would not be beneficial. Larvae of an insect dehydrated 20-30% at 5°C survived freezing much more poorly than did controls. Less ice formed at the -10°C freezing temperature but greater injury resulted.

The third and final principle of insect cold hardiness is freezing tolerance, i.e. survival in the presence of tissue ice for prolonged periods. This subject provides one of the more interesting extremes in the area of insect physiology, and the study of which will be the basis of this dissertation. More detailed descriptions are to be found in Chapters 2 and 3.

A large number of researchers have considered certain aspects of this problem but have only been able to generalize and disagree. The discovery of glycerol as a principal solute in various insects in the late 1950's along with the theories of protective action available (Smith, 1961), tended to confirm the cryoproctective role of glycerol. Confirmation came from the studies of Dubach, et al. (1959) and Salt (1957, 1958, 1961, 1962, and 1969). Asahina (1966) supported Somme (1964) in stating that "glycerol alone cannot protect against freezing injuries". This conclusion was based upon the observation that a few insects proved exceptions to the rule. That is, one insect larva contained no glycerol and yet was freezing tolerant while a number of insects

containing relatively high concentrations were freezing susceptible.

For the moment it should be mentioned that Somme may have failed to consider the specimens conditions with respect to previous history of temperature exposure. He also failed to understand that glycerol's protective action has limitations which are concentration dependent. Finally, Somme did not consider that in the freezing susceptible insects studied, glycerol was acting to protect via extensive depression of supercooling. Supercooling points as low as -49°C were measured; however, upon freezing, death resulted. Granted, glycerol did not protect against ice damage but its action was to enhance supercooling to temperatures well below potential ambient exposure.

Asahina (1966) while not rejecting the concept of glycerol affording cryoprotection in some insects, doubts that its presence alone was the sole factor responsible for frost resistance. This was a reasonable conclusion in light of the evidence that other cryoprotectants (i.e., sorbitol) naturally occur in insects. However, the reasons cited for his statement were less than convincing. Firstly, Somme studies (1964 and 1965) formed the principal basis for the conclusion. Secondly, Asahina believed that the work of Takehara and Asahina (1960) and Tanno (1963) substantiated this conclusion. These workers injected glycerol into freezing susceptible insects during diapause only to note that no freezing tolerance was acquired. They did not consider that glycerol in supra-physiological doses is lethal to most tissues. They also noted that normal development proceeded in controls but did not consider the possible dual action of low temperature and glycerol toxicity

in an insect not naturally exposed to these conditions. Upon freezing glycerol levels would effectively increase to even greater concentrations. Finally, the work was not adequately controlled since no studies were performed to determine the effects, if any, resulting from the injection trauma (mechanical) or to determine the effective dispersion of glycerol.

In light of these apparent questions and comments, the studies described in Chapters 1, 2, and 3 were undertaken. A specimen naturally exposed to extremely low temperatures, while containing high glycerol levels, was required for study. The carabid beetle, Pterostichus brevicornis, was known to hibernate in decayed stumps in the Fairbanks locale (Miller, 1968 and 1969). Exposure to low ambient temperatures during the Alaskan winter was suspected and hemolymph glycerol levels were found to be high during this period. This insect provided a system whereby the answers to a number of questions concerning freezing survival could be sought. These were, if glycerol is a cryoprotectant, what are its mechanisms of action? Is glycerol concentration related to environmental stimuli? In what ways does glycerol effect the physico-chemical characteristics of freezing and supercooling? What are the seasonal relationships between glycerol concentrations and these physico-chemical characteristics? What are the apparent sources of glycerol? What ecological factors are significant to cold hardening. Of what value would such an adaptation be to a given population? How are particular tissue systems, especially the nervous system, affected by freeze-thaw encounters? Finally, what

are the effects of low temperature on coordinated neuromuscular activities?

While these questions are numerous and diverse, they have explicit interrelationships. Many have been answered completely, others only partially and yet others only considered from theoretical standpoints.

Studies relating to the understanding of nervous function and neuromuscular coordination were undertaken in light of a rather interresting work. Burkett and Schneidermann (1968) described coordinated neuromuscular activity in "frozen" diapausing moth pupae. Not only did spiracular valves continue to function at -5°C but movement indicative of response to $PO_2$ and $PCO_2$ were noted. Other studies concerned with neural function (adaptation) in insects at low temperatures are not present in the literature. If sustained activity reflected both behaviorally and neurophysiologically could be demonstrated, it would be of immense value to the insect with regards to survival. Such activities while in the supercooled state could prolong feeding, development, etc., thereby conferring an adaptative advantage in the insect. Chapter 4 will discuss such an adaptation.

# CHAPTER 1

## GENERAL ECOLOGICAL, ETHOLOGICAL AND PHYSIOLOGICAL ASPECTS OF OVERWINTERING

Life History and Microhabitat

As previously mentioned, Pterostichus brevicornis (subgenus Cryobius) was the principal species studied. This carabid beetle is known to have a circumpolar distribution generally restricted to latitudes above 54°N. However, two other isolated refugia are represented: the northern coastal Great Lakes region and the eastern North American area (Ball, 1963). The life history of this insect has been discussed in depth by Kaufmann (1970) while Miller (1969) has considered aspects of its temperature survival. Both of these studies were conducted simultaneously to the experiments presented in this dissertation and are therefore directly related.

The general problems faced by arctic and near-arctic insects have been considered in depth by numerous investigators (Bertram, 1935; Mason, 1958; and Downes, 1965). The major limiting factor is obviously temperature. Prolonged winters with sub-freezing temperatures for nearly three-fourths of the year followed by short, cool summers restrict all but the hardiest forms. Coupled with temperature are the factors of light patterns (total daylight in summer vs. constant darkness in winter) and suitable habitat availability (restricted flora and food sources).

P. brevicornis has been found to adapt in a number of diverse ways that tend to aid in its survival in the north. Adult and larval stages have been found both in summer and winter. The summer residence for both groups is predominently the mossy floor of the taiga while winter adult stages are found in decayed stumps and fallen timber. An apparent preference for spruce (Pices mariana) exists but both birch (Betula papyrifera) and cotton wood (Papulus balsamifera) stumps may also be utilized. Habitats

must contain crevices or tunnels, be decayed to a fibrous state and moist.

Following the first frost, the adults aggregated in stumps in ever increasing numbers. Individual groups of greater than 100 beetles per stump were not uncommon, however, isolated individuals were also located. Migration into the stump was both vertical and horizontal and apparently stimulated by either the gradual decrease in niche temperature or the degree of thermal fluctuation, i.e. to a certain low temperature. The biological significance of these aggregations remains unknown. Certainly there would not be an immediate thermal advantage over ground conditions but close proximity of breeding individuals during arousal may be important. The possibility also exists that these aggregations were accidental and dependent upon the stump structure, i.e. available channels and chambers. However, it is believed that this is not the case due to the relatively immense selection of habitats presented to this insect. Also, a strong, odoriferous secretion from the pygidial glands was readily detected either on handling or crowding. While these glands are thought to function primarily in defense, the question of olfactory stimulation being similar to that of trail marking in ants may be raised.

Total life span within this species was variable. Kaufmann (1970) found that it varied between 14 and 36 months; immature stages - 2 to 12 months and adults - 12 to 24 months. All developmental stages excluding eggs were found to overwinter.

In the same study Kaufmann noted a number of surprising observations which indicated that hibernation within this insect was not a state of

complete physiological inactivity. These observations were:

(1) While frozen at ambient temperatures mostly below -20°C, egg development was initiated (January) and continued into spring. Ovaries had been completely empty at the beginning of the hibernating period.

(2) Evidence for digestive processes continuing through winter was obtained. Upon entry into the hibernacula, the alimentary canal was filled with food. Through the early phases of overwintering the tract became emptied. Mean ambient temperatures approximated -10°C and presumably a large majority of the population had frozen earlier.

(3) Feeding resumed during late January and continued during warm spells (above -5°) thus demonstrating active feeding behavior while supercooled.

(4) Finally, size of fat body was found to oscillate throughout winter paralleling changes in ambient temperatures and in inverse relationship to glycerol concentrations (see Chapter 2). Thus, indicating substantial variations in metabolic processes at temperatures well below freezing ($\sim$ -10°C) and possibly to temperatures as low as -40°C to -50°C.

Seasonal variations in ability to withstand freezing temperatures have been considered by Miller (1969). He noted that summer specimens were completely susceptible to freezing below the supercooling point, but freezing tolerant throughout winter. Cooling rates and exposure durations were found to be critical as were rates of rewarming. Cooling rates of

20°C per hour or less favored nearly complete survival. For example, specimens cooled at 20°C per hour to -30°C demonstrated 100% survival while those cooled at 4°C per hour to -70°C demonstrated 90% survival. Exposure durations were 1 and 2 hours respectively. It should be noted that survival criteria were extremely stringent. Beetles had to be capable of directed, coordinated activities (walking, feeding, etc.) and injury free four days following testing.

Water content was also determined throughout the above series and found to vary between a mean summer high of 65% and a mean winter low of 54%. This is a relatively small difference and not sufficiently large enough to account for the observed depression in freezing points.

Thermal Fluctuations Within the Microhabitat

In light of these observations and those to be discussed in succeeding chapters, some indication of the actual habitat exposure temperatures is required. The acquisition of continuous temperature records of the hibernacula was logistically unfeasible at the collection sites whereas compliation of daily ambient (air) temperature fluctuations was possible. These records were of sufficient accuracy to reflect gross temperature changes during winter at ground level and within the stump provided no blanket of insulative snow was present. On numerous occasions individual winter stump temperatures were measured and found to be in close accord with those of air, and even colder at times reflecting both previous exposure temperatures and the "frost-front" migration (Bertram, 1935). Summer stump temperatures were relatively stable, ∿10°C to 15°C,

but of little relevance since the beetles were on the forest floor.

Although the exposed stumps reflected air temperatures, they acted as an insulative buffer to extreme or rapid fluctuation in temperature. This damping action was directly dependent upon depth. Figure 1-1 illustrates this feature. A stump containing a horizontally placed thermal gradient probe was stabilized at various depths at the temperatures indicated by time 0. At this point the stump was placed in a freezer held at -42.5 $\pm$ 2.5°C and a temperature record obtained each hour at each centimeter in depth. A tabulation of cooling rates derived from the initial temperature (time 0) to -30°C at each depth is found in Table 1-1. At a depth of 1 cm cooling occurred at the rate of 25°C per hour but was decreased five-fold just 1 cm further in depth to 5°C per hour. Between 2 cm at the center, there was only a two-fold decrease in cooling rate.

While such an initial gradient would certainly not be encountered in the wild, it can be seen that the stump would buffer extreme temperature variations but would within hours reach the new low temperature, thereby allowing the insect to experience a gradual temperature change. One field observation may be mentioned at this point. Extreme variations in the microhabitat were occasionally experienced and survived by *P. brevicornis*. On a January day with air temperature at -40°C, the subbark temperature of an exposed, well insolated stump was 0°C. Beetles were found active and slowly walking. When the stump was suddenly shaded by a cloud, the temperature dropped to ambient within 1-2 minutes. The insects froze but upon artificial rewarming, appeared healthy.

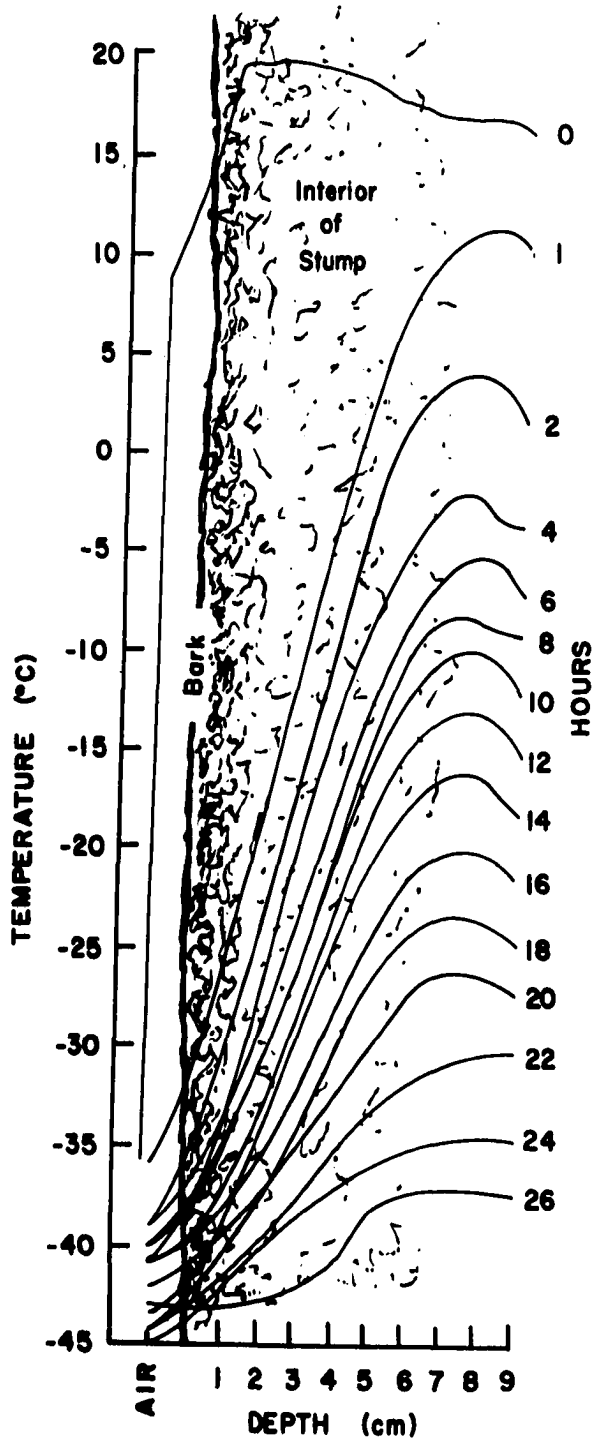


Figure 1-1. Temperature changes at various depths (cm.) in a rapidly cooled decayed stump hibernaculum. Time 0 line represents initial temperatures.

TABLE 1-1

Cooling rates of decayed stumps per centimeter depth (horizontal) upon acute cooling to -30°C. Time 0 temperatures ranged between 18 and 20°C.

| Depth (cm.) | Cooling Rate (°C/hr.) |
|---|---|
| Bark | 100 |
| 1 | 25 |
| 2 | 5.2 |
| 3 | 3.5 |
| 4 | 2.8 |
| 5 | 2.4 |
| 6 | 2.3 |
| 7 | 2.1 |

The buffering effect of the insulative stump is graphically illustrated in Figure 1-2. This stump as in Figure 1-1 was taken from room temperature (18 to 20°C) and placed in a freezer at ∿ -42°C for 27 hours. Oscillations in air temperature were evident, however, the amplitude of these variations were dampened by a depth of only a few centimeters. Each numbered line represents the respective depth of a thermocouple within the stump.

The same system was acutely warmed after the above cooling procedure to determine if any difference existed between cooling and warming rates. Figure 1-3 illustrates the rates encountered upon rewarming. As in Figure 1-2 each curve represents a given depth (cm) of a thermocouple within the stump. On comparing Figure 1-2 and Figure 1-3, it is obvious that the cooling and warming rates were strikingly different, an observation not readily expected. In calculating a number of rate differences, it became evident that the stump warmed (upon reversal of the temperature gradient) exactly twice as fast as it initially cooled. For example, at a depth of 4 cm, cooling occurred at 20°C per hour until freezing while warming to thawing occurred at 40°C per hour. At a depth of 7 cm, cooling to freezing progressed at 13°C per hour while warming to thawing progressed at 25°C per hour. The explanation of this observation is apparent if it is recalled that these stumps were very moist (high water content). The specific heat of the "stump water" may be estimated to be 1.0 cal $degree^{-1}gm^{-1}$ near freezing and the specific heat of ice after freezing estimated to be 0.5 cal $degree^{-1}gm^{-1}$. Therefore upon cooling, twice the energy output (heat loss) would be required per gram of stump to reach freezing temperatures.

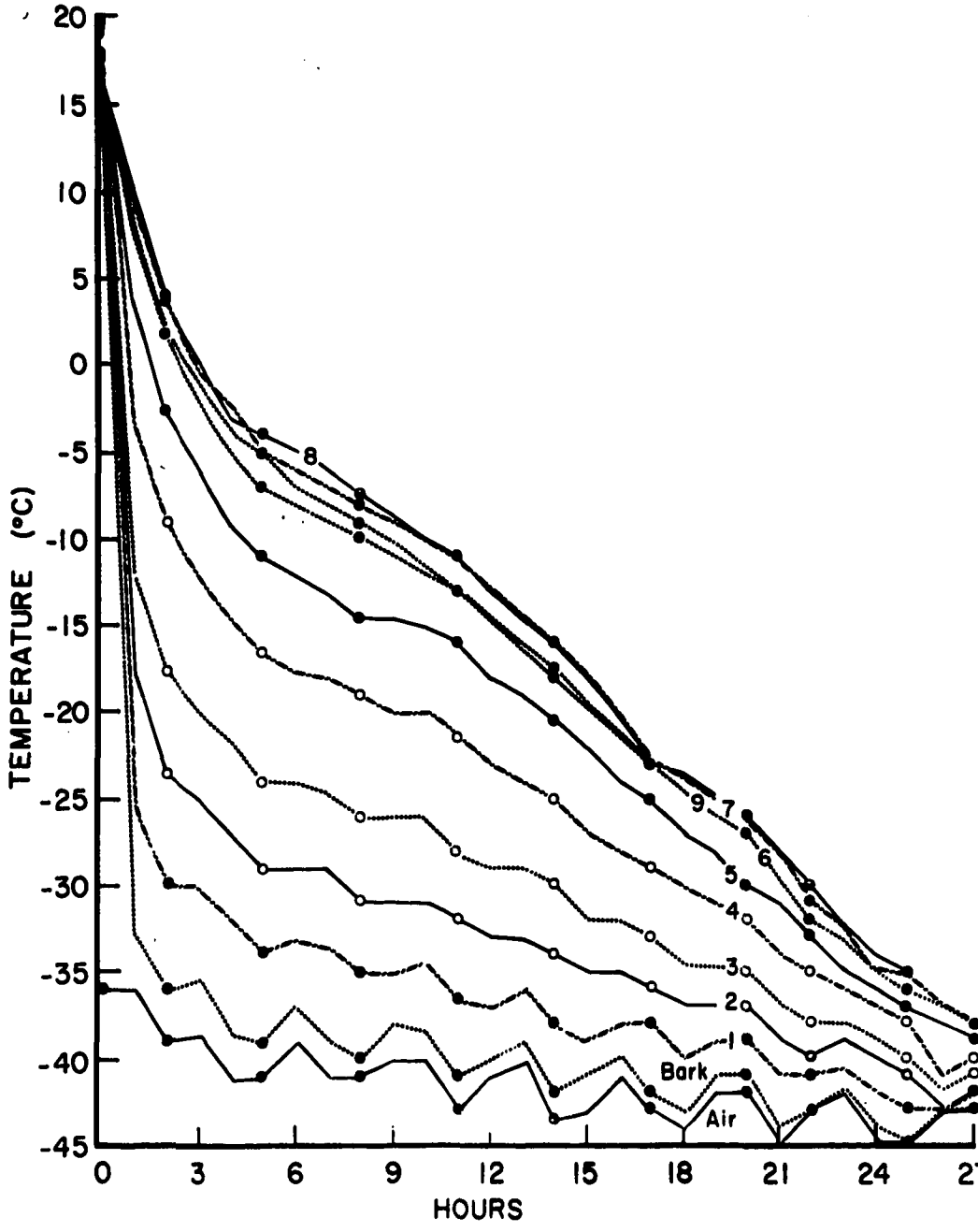


Figure 1-2. Cooling rates of stump at various depths (cm.) upon artificial exposure to -42° ± 2°C. Each line (#1-9) represents horizontal depth of thermocouples.

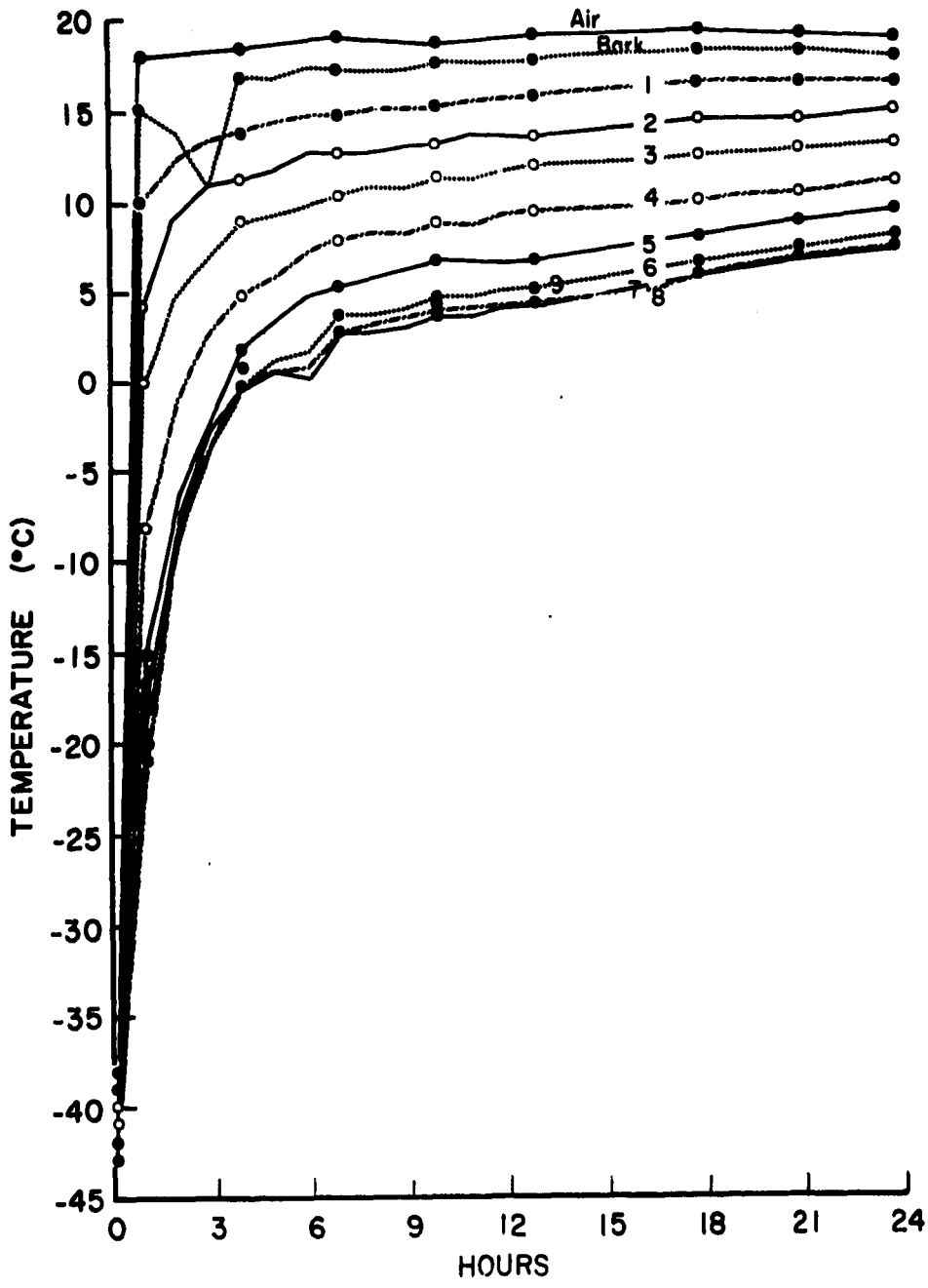


Figure 1-3. Warming rates of a stump at various depths (cm.) upon exposure to air temperature of 18°C. Each line (#1-9) represents horizontal depth of thermocouples.

That is, warming to thawing would require half the heat input per degree per gram of stump than the heat loss per degree per gram required to initially freeze the stump.

These thermodynamic considerations may be considered in an ecological sense as the stump offering an ideal hibernaculum to insects for a number of reasons. First, fluctuations in ambient temperature would be reduced (dampened) due to the insulative properties of porous wood. Second, rapid decreases in ambient temperatures while ultimately reflected in the stump would be reduced, i.e. cooling rates would be diminished. Third, upon warming, the stump interior would reach thawing temperature at approximately twice the initial cooling rate. Figure 1-4 represents varied cooling and warming stages of a stump and graphically illustrates the three effects in total.

Modifications in thermal factors resulting from snow cover have not been considered. It is common knowledge that snow is an effective insulating material. Ambient temperatures as low as -35°C have been recorded while ground level temperatures were -3.1°C, a 32°C gradient through 45 cm of snow. However, throughout the three winters studied, most specimens were collected from exposed stumps (above snow line). A few periods existed during which snow cover had profound influence on temperature exposure thereby affecting changes in cold hardiness. These periods will be discussed in Chapter 2.

## Locomotor Responses to Temperatures

The survival advantages gained by the stump dwelling beetle were

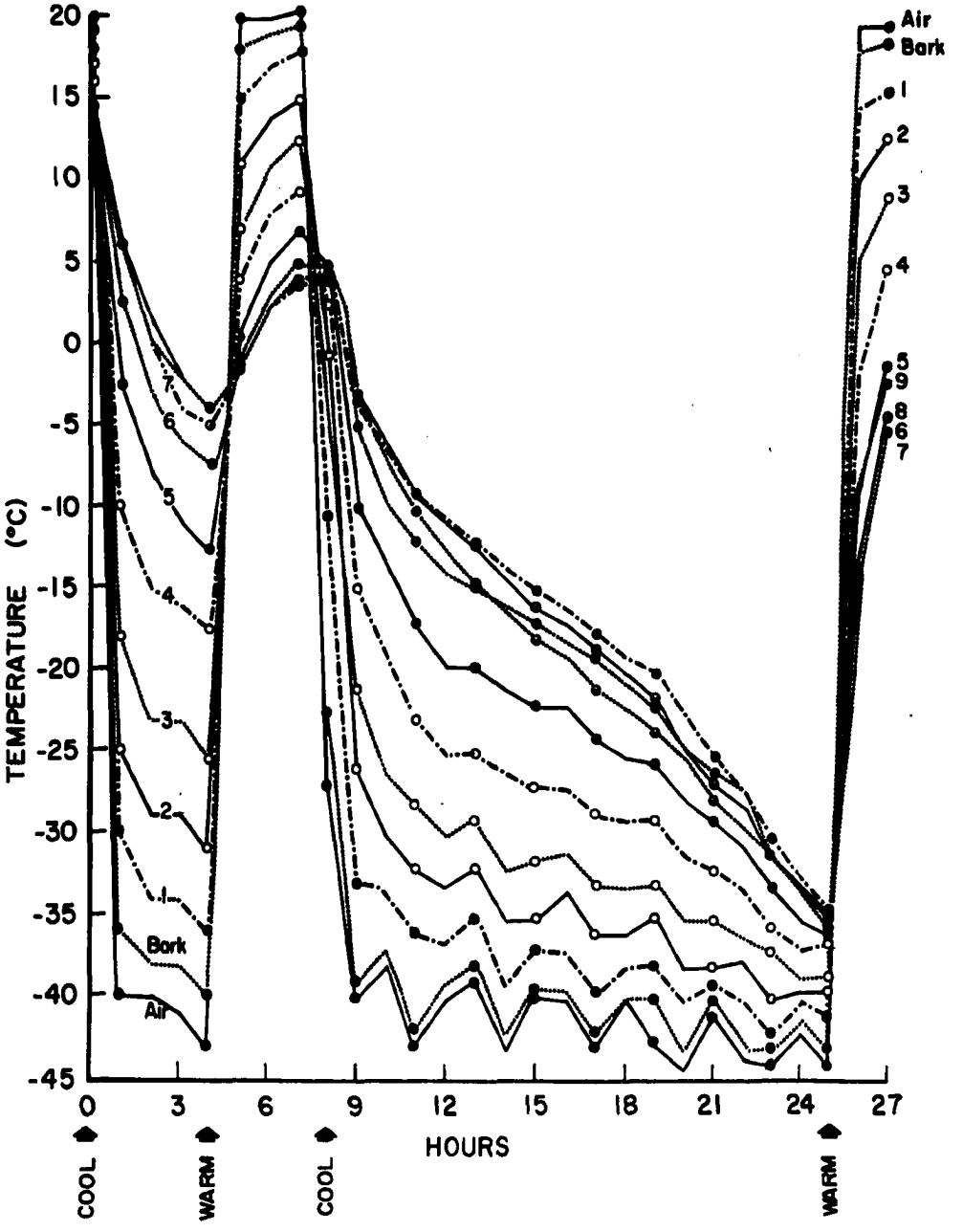


Figure 1-4. Variations in stump temperatures effected by oscillatory warming and cooling. Each line (#1-9) represents horizontal depth (cm.) of thermocouples.

alluded to in the previous sections. However, demonstration of the beetle's ability to respond to varying thermal stimuli has yet to be considered. If an insect is to benefit from the slow cooling and fast warming characteristics of the stump, it must be able to respond to these temperature variations. In a sense, migrations have been observed. The initial movement to the stump occurred during early fall after first frost. During this period most beetles were found near the stump periphery. As fall and winter progressed, the beetles were found successively deeper in the hibernacula, provided sufficient passages were available. This observation indicated that movement occurred during the supercooled state prior to freezing and after occasional winter thawing.

In order to test this observation a temperature gradient chamber was constructed. Figure 1-5 illustrates the structure of this unit. A glass tube, diameter 3", was sectioned off for an effective length of 100 cm. Thermocouples were placed on the bottom surface every 5 cm. Each end of the chamber was sealed and a one inch aluminum rod passed through the center. One end of the rod passed into a heat exchanger cooled the -17°C while the opposite end abutted against a 50W heat source (light bulb). The entire tube was insulated with one inch of black foam rubber. Three entry ports were placed in the chamber top and stoppered with moist cotton. The gradient could be adjusted to any desirable range but was eventually maintained between -12° and +20°C. Fifteen to twenty outdoor specimens were placed in the chamber through the warm port to insure against the possibility of initial cold trapping. Temperature preference runs were conducted in darkness while a relatively high humidity was maintained by

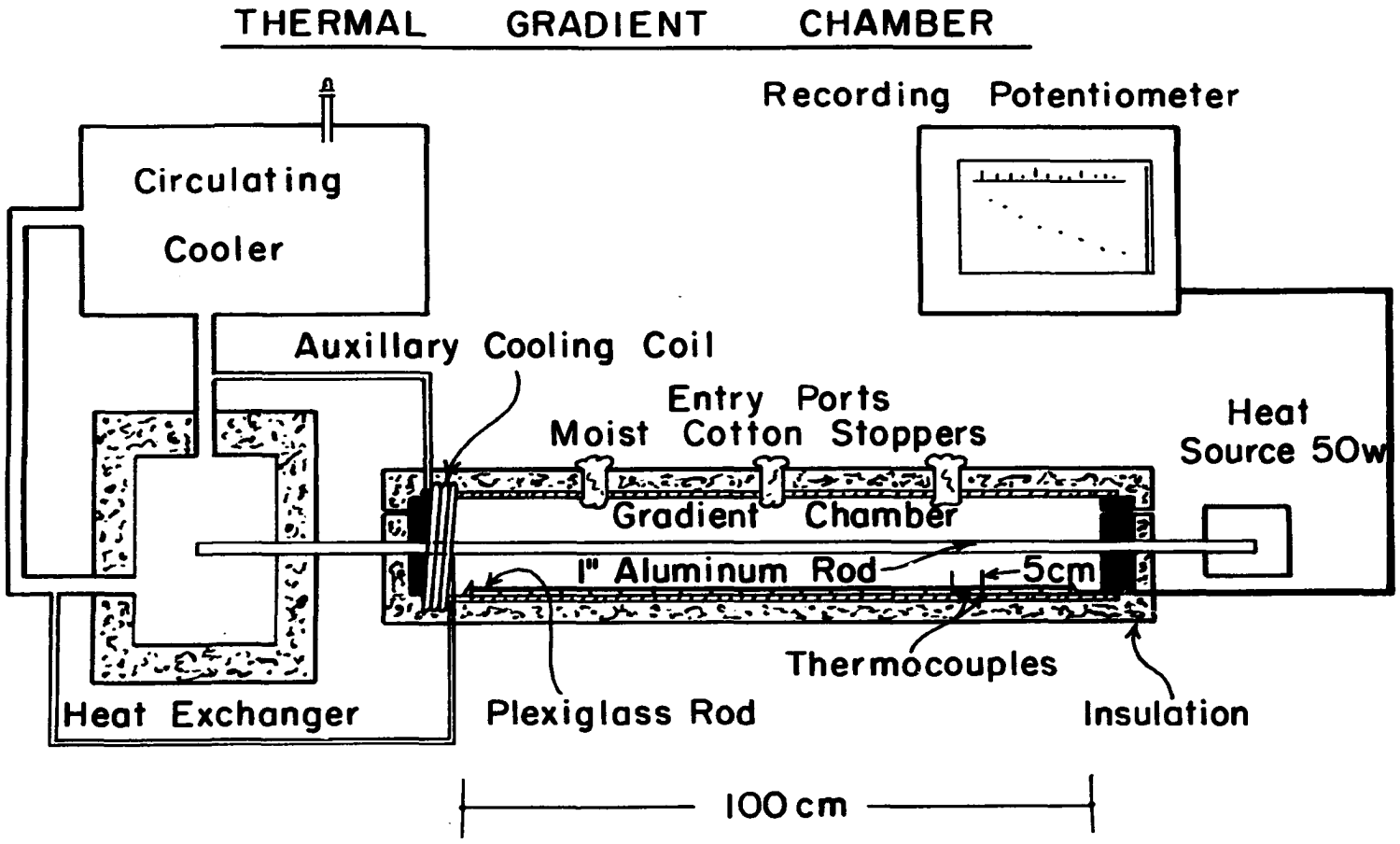


Figure 1-5.

wet cotton stoppers. Humidity was judged to be near absolute (saturated) by the increased levels of condensation on chamber walls at below freezing temperatures and by the moisture accummulation on the walls at the above freezing end. Preferences were determined for each insect after 24 hours in the chamber. A 24-hour period was chosen since initial observation indicated that many specimens continued to wander throughout the first 15-18 hours in the gradient.

Figure 1-6 illustrates the results of the thermal preference experiments as determined over a one and one-half year period. Plotted are mean (circle) and individual (black bar) preferences and standard deviation of the mean. Temperature preferences varied from a winter low of -5.5°C to a summer high of 13.3°C. The former temperature is within the lower limit of mobility. These data provide interesting assessments of mobility limits in the winter beetles. Some insects have demonstrated continuous and coordinated activities at temperatures close to their supercooling points, especially in winter, thus indicating an ability to migrate deeper into the stump on occasional winter thawing.

Details of glycerol variations will be discussed in the succeeding chapter, however, it is important to note one observation at this time. A plot of glycerol concentration vs. mean seasonal temperature preference demonstrates that temperature preferences have been found to be below the summer hemolymph freezing point (-0.6°C) only in those specimens possessing glycerol (Fig. 1-7). While the comparative data are sparse, it can be noted that an apparent linear correlation (-0.80) between glycerol concentration and temperature preference existed if summer beetle data were omitted.

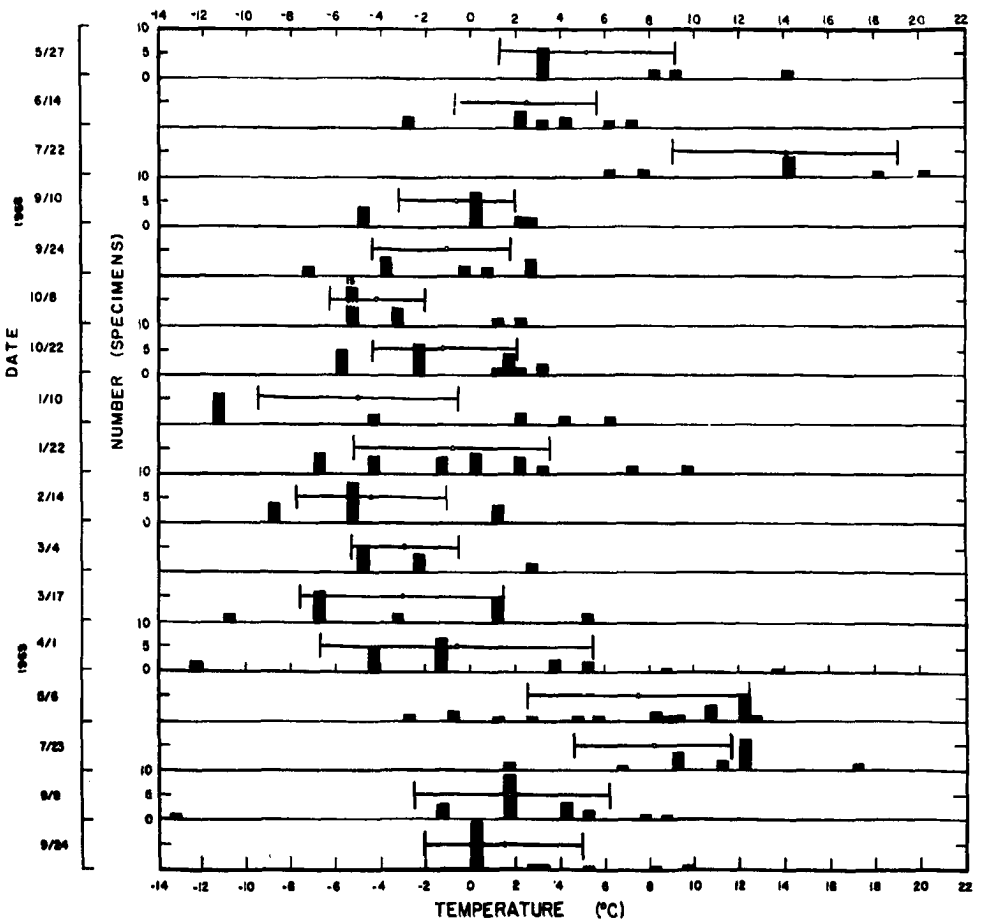


Figure 1-6. Seasonal variations in individual and mean temperature preferences demonstrated by adult P. brevicornis. Mean values are ± standard deviation.

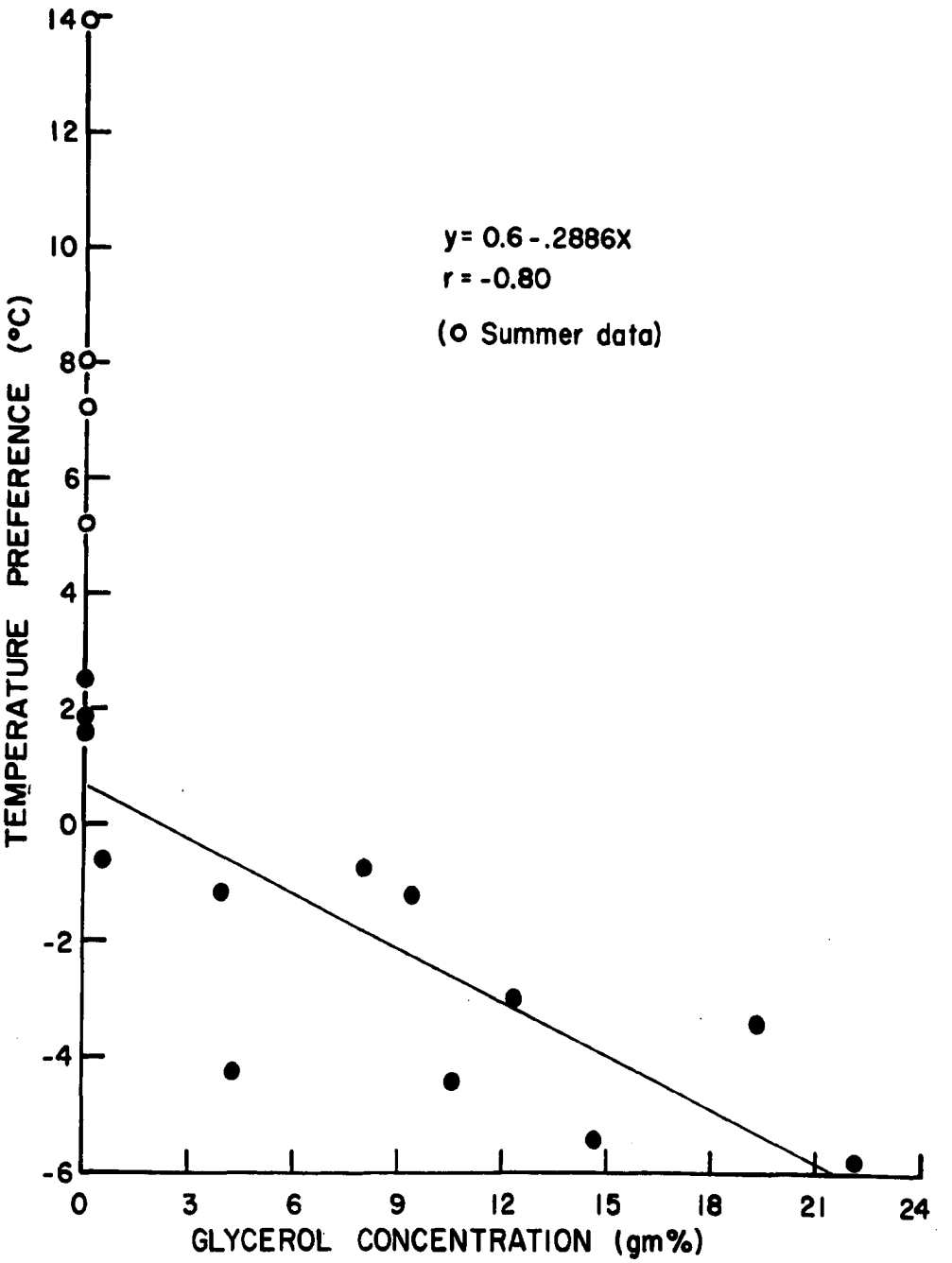


Figure 1-7. Linear regression plot of glycerol concentration vs. mean seasonal temperature preferences in P. brevicornis. Summer data (0) were excluded from statistical interpretation (r= correlation coefficient).

The relevance of such a figure may be questionable; however, it does indicate a trend. Namely, P. brevicornis will actively avoid freezing conditions prior to attaining a suitable level of cold hardiness.

# CHAPTER 2

## VARIATIONS IN COLD HARDINESS AS RELATED TO GLYCEROL

# INTRODUCTION

Early attempts to correlate freezing survival in insects with a cryoprotective substance such as glycerol have provided inconclusive evidence and even dubious results (Somme, 1964 and Asahina, 1966). The simplistic idea that if an insect contained such a compound independent of the considerations of concentration and general physiological state, it should behave as an automaton and survive all rigors that the experimenter could conceive, fostered such results. It was not until an in depth understanding of the modes in which glycerol afforded its protective influence within insects was realized (Salt, 1961) that new insights were gained into the question of freezing survival. To recapitulate briefly, a cryoprotectant acts in insects either (1) to increase frost resistance by greatly lowering freezing and supercooling points but without affording protection in the event of ice formation or (2) to allow varying degrees of freezing protection without necessarily profound lowering of freezing and supercooling points.

The former mechanism is frequently demonstrated in most insect developmental stages and is thought to be associated with specific behavioral responses resulting in non-feeding prior to hibernation. This results in the reduction of nucleating agents within the gut thereby lowering supercooling points (Salt, 1968). The later mechanism is evident within insects that retain normal activities (temperature limited), such as feeding in spite of low ambient temperatures but within the supercooling range. Discussions of this type of adaptation are wanting save Baust and

Miller (1970). It will be the object of this section to discuss such an adaptation in depth.

## METHODS AND MATERIALS

Pterostichus brevicornis, a carabid beetle (average weight 8-9 mg) which overwinters in both the adult and larval stages was the principal subject of study. Other members of this genus were in all probability included in most analyses although in very small numbers. However, due to similar habit and other ecological considerations along with similar physiological responses, it is believed that little significant differences were encountered.

Ecological considerations have been discussed in the preceding chapter including probable thermal exposure regimes. Acclimatization studies utilized outdoor specimens while acclimation experiments were conducted in temperature regulated environmental chambers.

Glycerol content in the hemolymph was determined utilizing a chromatographic technique described by Perkins and Aronoff (1959) and modified by Somme (1964). Ascending chromatograms were run on Whatman No. 1 paper (20x40 cm) with a single phase solvent, n-butanol:glacial acetic acid:water (12:3:5 v/v). Hemolymph was collected with a one microliter syringe (Hamilton Co., Whittier, California) supported in a micromanipulator. The samples as well as standards were then applied to the paper. Chromatograms were run for 18 hours at 20°C. After air drying, chromatograms

were sprayed with a solution of 0.01m aqueous potassium periodate, dried and sprayed with a solution of 35% saturated sodium tetraborate (v/v), 0.8% potassium iodide (w/v), 0.9% sodium tetraborate (w/v) and 3% soluble starch (w/v). Glycerol and other polyhydroxy alcohols yield white spots on a blue-brown background following this treatment (Fig. 2-1). Table 2-1 lists the relative Rg values of various polyhydric alcohols in the above solvent system and indicates a lack of interference with glycerol identification.

Detection limits of this method are better than 1.0%. That is, a standard solution containing 10μg glycerol per 1.0μl is resolvable. Sample concentrations were determined by reference to a standard curve constructed by plotting weight of excised spots against the standard concentrations. This curve was linear for standards ranging from 2 to 60% glycerol (w/v) (Fig. 2-2). Spot size was dependent upon absolute amount of glycerol applied and independent of carrier solvent volume.

Each determination is representative of a pooled hemolymph sample ranging in volume between 0.75 and 1.00μl. In general, hemolymph from 3-5 specimens was required to collect this volume. This quantity of hemolymph was utilized because it was felt that 1.00μl approximated the total blood volume. Such a supposition was based upon the conclusions of Altman and Dittmer (1961) and Wheeler (1962). They found that total body hemolymph generally accounted for approximately 12-18% of the insect's weight. One microliter of hemolymph from Pterostichus weighs slightly less than 1.1mg while mean total body weight ranges between 8-9 mg.

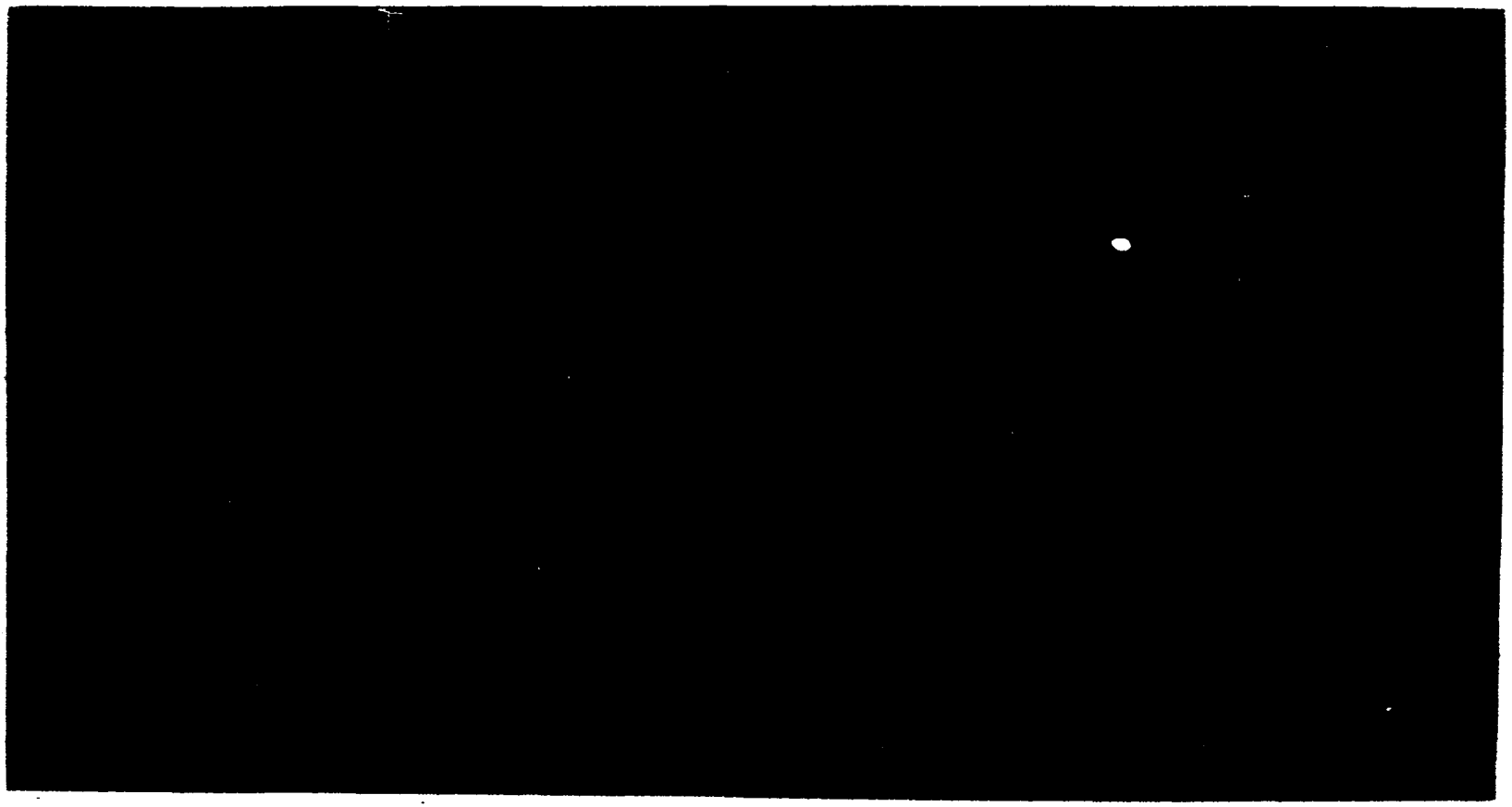

Figure 2-1: Illustrates the migration of glycerol vs. various polyhydric alcohols (sugars) in butanol-glacial acetic acid=water (12=3=5). Ascending chromatograms were run on Whatman #1 paper. A Glycerol standard was applied at the origin with each polyol.

| | | | | | | |
|---|---|---|---|---|---|---|
| 1=Mannose | 3=Sorbitol | 5=Fructose | 7=Dulcitol | 9=Inositol | 11=Xylitol | 13=Adonitol |
| 2=Mannitol | 4=Glucose | 6=Sucrose | 8=Galactose | 10=Trehalose | 12=Erythritol | 14=Arabitol |

TABLE 2-1

Rg* values from chromatograms of various cryoprotective compounds (sugars and polyhydric alcohols).

| Compound | Rg value |
|---|---|
| glycerol | 1.00 |
| adonitol | 0.40 |
| arabitol | 0.38 |
| dulcitol | 0.22 |
| erythritol | 0.63 |
| fructose | 0.33 |
| galactose | 0.15 |
| glucose | 0.20 |
| inositol | 0.03 |
| mannitol | 0.22 |
| mannose | 0.26 |
| sorbitol | 0.26 |
| sucrose | 0.07 |
| trehalose | 0.00 |
| xylitol | 0.35 |

Solvent n-butanol:glacial acetic acid:water (12:3:5v/v)

*Rg = migration of compounds relative to glycerol migration.

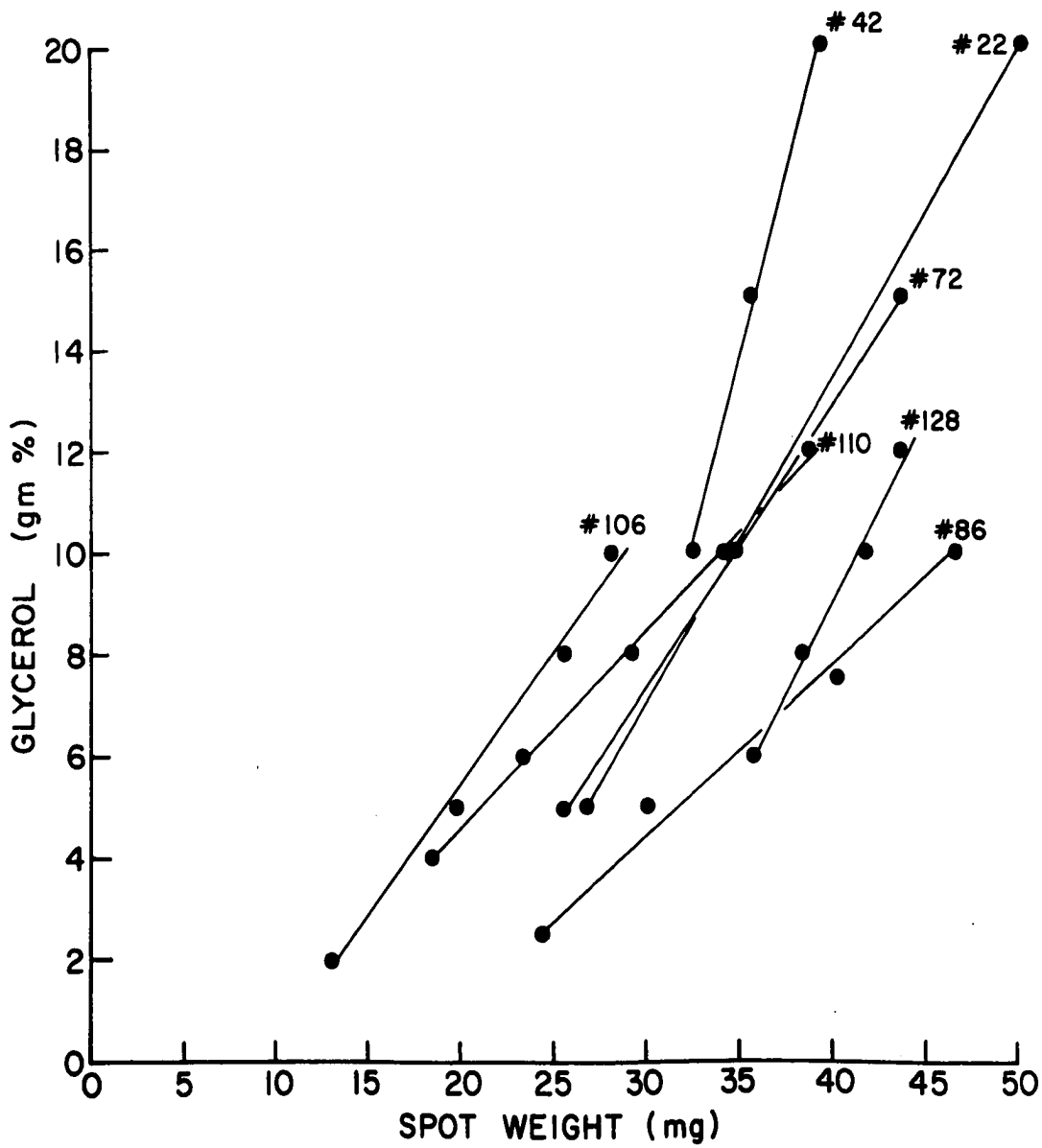


Figure 2-2

Supercooling points were measured with a 32ga. copper-constantan thermocouple on the abdomen while cooling at 1 or 3°C per minute. Temperatures were indicated on a recording potentiometer (Leed and Northrup Co., Model G). Initiation of spontaneous freezing resulted in termination of supercooling and is indicated by a rise in the temperature curve due to released heat of fusion. The temperature at which spontaneous freezing occurred was termed the supercooling point. Surface contact between the thermocouple and cuticle was insured by using a small dab of petroleum jelly.

Hemolymph freezing points in the non-supercooled insect were measured using a modified Scholander freezing point apparatus (Fig. 2-3). Hemoplymph samples were extracted rapidly with a microliter syringe, injected into a capillary tube, sealed at one end and covered with liquid petrolatum (mineral oil) and "flash" frozen. Samples were covered with oil to prevent evaporation and possible clotting due to prolonged exposure to air. The freezing point was defined as the temperature at which ice crystals in hemolymph remained constant in size, with neither growth nor melting being observed. This was accomplished by varying the bath temperature within the chamber.

Freezing points of various saline and glycerol standard solutions were not changed whether or not an oil cover was applied. Glycerol, inorganic salts and most organic compounds (fats, proteins and carbohydrates) are insoluble in liquid petrolatum.

Glucose concentrations of the hemolymph were determined utilizing thin-layer chromatography. Standard and unknown samples were run on

commercially prepared silica gel plates (Eastman Chromagram 6061). Plates were pretreated in a 2% solution of sodium bisulfite in 60% ethanol (w/v), dried and activated at 100°C for 15 minutes. The migrating solvent was ethyl acetate:methanol:glacial acetic acid:water (12:3:3:2 v/v). Visualization was accomplished by spraying the dried sheets with a solution of 5% aniline hydrogen phthalate in glacial acetic acid (w/v) and heating at 85°C. Zone detection (glucose spot) was made under long wave UV light (3660 Å). Glucose $R_f$ values were 0.33. Detection limits were less than 0.1% (w/v). That is, $10^{-6}$ g of glucose was easily resolvable. The blood sugar trehalose did not react with the indicator spray.

Hemolymph trehalose determinations were made utilizing a colorimetric technique devised by Wyatt and Kalf (1957). This procedure takes advantage of the exceptional stability of this sugar to both acid and alkali. Standard samples containing 50, 100, 200, 400 and 600µg of trehalose and unknowns (1µl each) containing trehalose were evaporated to dryness in pyrex tubes. The residue was then dissolved in 0.2ml of 0.1N $H_2SO_4$, capped with foil and boiled at 100°C for 10 minutes. This step hydrolysed any sucrose or glucose-1-phosphate. The solution was next cooled to room temperature and made alkaline with 0.15ml of 6N NaOH and heated to 100°C for 10 minutes. This results in the destruction of reducing sugars. The samples were then chilled in an ice bath for two minutes followed by transfer of 0.2ml of sample to a cuvette containing 1.5ml anthrone reagent (0.2% anthrone solution in 95% $H_2SO_4$). Samples were allowed 30 minutes to react and the optical density was determined in a Beckman DK-2 Recording Spectrophotometer at 590mµ (Fig. 2-4). Peak

# FREEZING POINT DETERMINATION APPARATUS

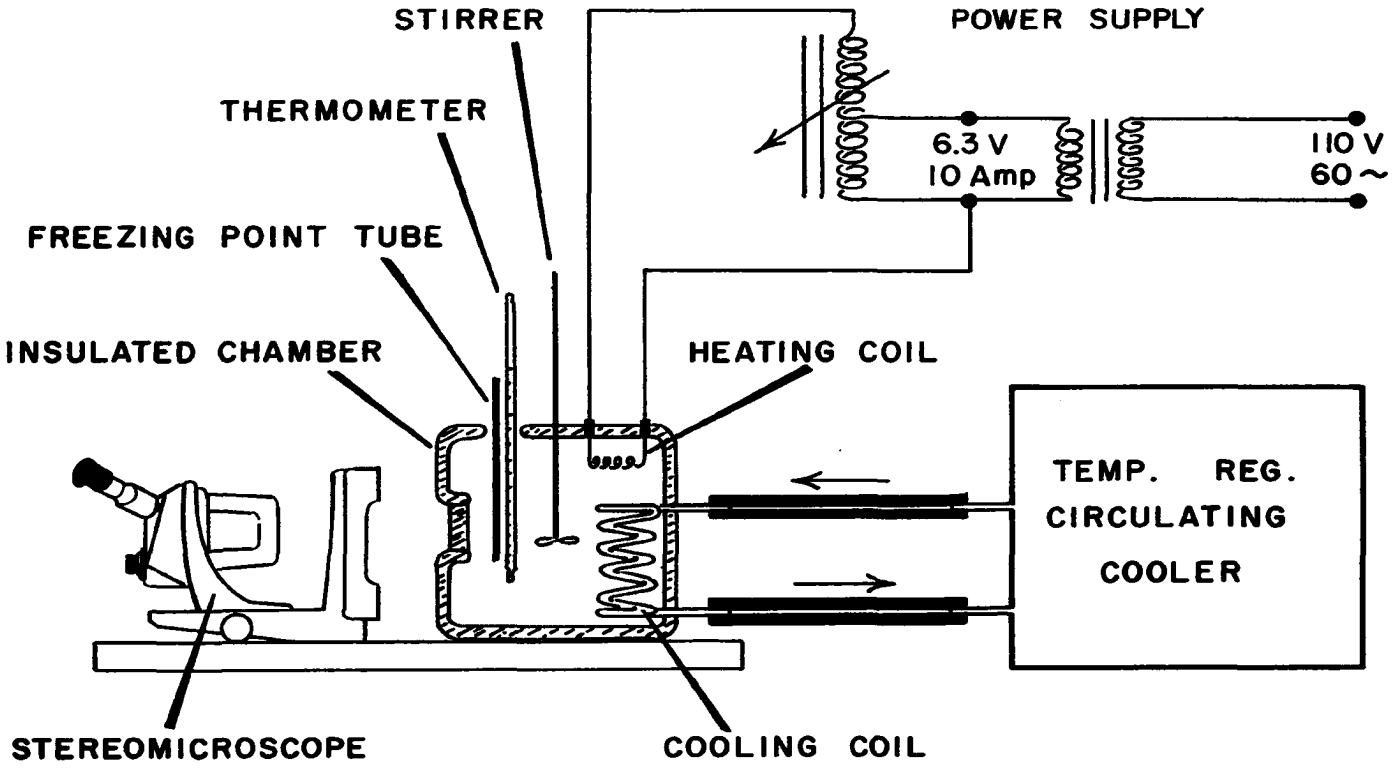


Figure 2-3

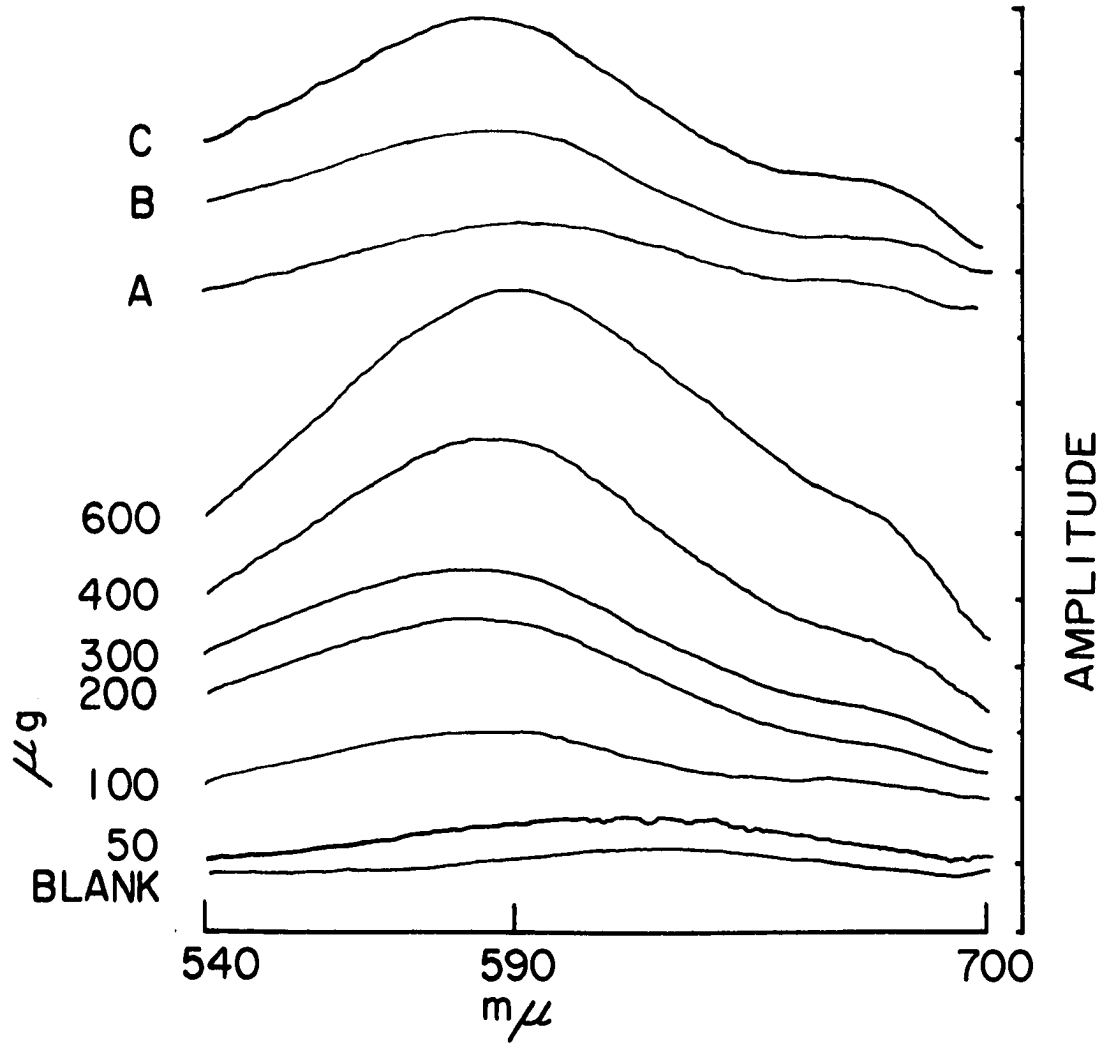


Figure 2-4: Redrawn chart from recording spectrophotometer illustrating the instrument's response to varying trehalose concentrations (standards, µg, and unknowns, A, B and C).

amplitudes of the standard curves were plotted against trehalose concentration. Trehalose concentration of the unknown hemolymph sample was extrapolated from this curve (Fig. 2-5).

## RESULTS

### Thermal Acclimatization Experiments

Glycerol concentrations in hemolymph varied seasonally as did supercooling points and hemolymph freezing points (Table 2-2). Figure 2-6 illustrates the temporal relationships between supercooling and freezing points and glycerol concentration.

Glycerol concentrations were observed to increase during fall and winter following the first frost both in 1968 and 1969. The initial increase appeared to be temperature related (Fig. 2-7) with continued fluctuations during winter (December-March) also apparently temperature dependent. As glycerol concentrations increased (August through December, 1968), supercooling and freezing points decreased. Glycerol content over the two year period correlated well with both the freezing and supercooling points. Linear correlation coefficients are $r = -0.76$ (freezing points) and $r = -0.84$ (supercooling points) (Fig. 2-8). These values are very significant for biological systems.

As glycerol concentrations decreased at winter's end, supercooling points increased. During this same period cold hardening expressed as lethality was decreased (Miller, 1969). Following the complete loss of

glycerol in May, supercooling points were observed to rise continually into mid-summer (July). Freezing points followed the same course. During the late summer period, the parameters of freezing and supercooling proceeded to decrease without any apparent stimulation to glycerol synthesis. These changes were minor being in the order of 1-2°C for supercooling points and 0.1-0.2°C for freezing points. Upon the initiation of glycerol synthesis, these points were rapidly lowered.

The plot of mean freezing points was a near mirror image of the glycerol content curve (Fig. 2-6). The relationship was linear over most of the year with mid to late winter excepted. This latter time reflects a period of glycerol fluctuation without the expected changes in freezing points. In general hemolymph freezing points were depressed 1°C per 4gm% glycerol increase. The relationship between mean glycerol concentrations and supercooling points as illustrated in Fig. 2-8 was close to linear. Total body supercooling points were depressed 0.9°C per 4gm% glycerol increase. That is, the freezing point depression was nearly equal to that of the supercooling point depression with the same incremental change in glycerol. However, since supercooling is a statistically random phenomenon dependent on variable factors such as time at a given low temperature and rate of temperature decrease (assuming constant amounts of nucleators), there is no reason to necessarily expect a perfect 1:1 relationship (Salt, 1961 and personal communication).

During December and January (1968-1969), glycerol concentrations were observed to decrease followed by an increase during February and March. However, the range of supercooling did not fluctuate significantly,

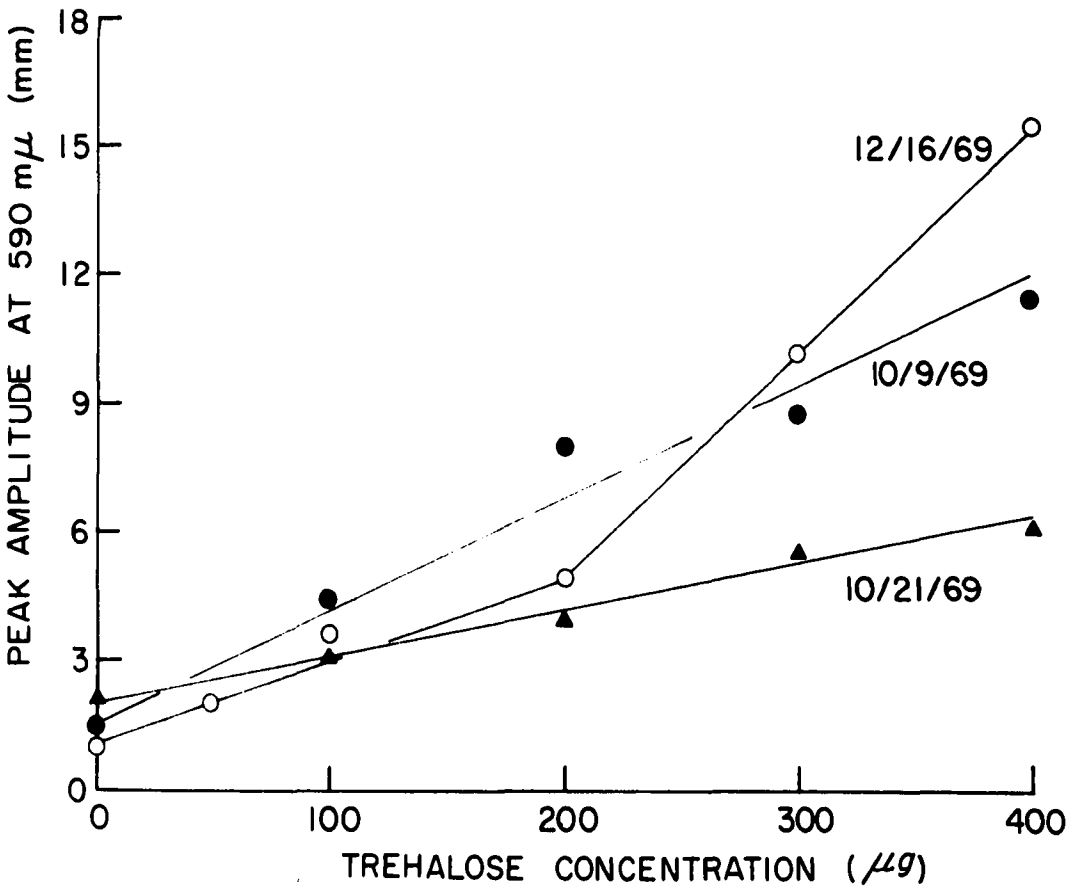


Figure 2-5: Sample standard curves of trehalose concentration vs. peak amplitude at 590 mμ.

TABLE 2-2

Seasonal changes in glycerol content, supercooling and freezing points of naturally acclimatized Pterostichus brevicornis.

| Date | Glycerol conc (gm%) | Supercooling Points (°C) | Freezing Points (°C) |
|---|---|---|---|
| 1/3/68 | 17.8% ± 0.8 | -9.0 | - |
| 1/22/68 | 16.5% ± 2.5 | -9.7 | - |
| 2/27/68 | 23.0% | -9.4 ± 0.3 | - |
| 3/9/68 | 21.0% | - | - |
| 3/25/68 | 18.0% ± 0.0 | -8.3 | - |
| 4/30/68 | 1.5% | -8.1 | -3.0 ± 0.0 |
| 5/14/68 | 0% ± 0.0 | -6.0 ± 0.4 | -3.0 ± 0.0 |
| 5/28/68 | 0% ± 0.0 | -6.8 ± 0.3 | -1.4 ± 0.0 |
| 6/11/68 | 0% ± 0.0 | -6.0 ± 0.0 | -1.1 ± 0.1 |
| 7/5/68 | 0% ± 0.0 | -4.6 ± 0.1 | - |
| 7/23/68 | 0% ± 0.0 | -4.2 ± 0.6 | - |
| 7/29/68 | 0% ± 0.0 | -4.7 ± 0.5 | - |
| 8/5/68 | trace | -5.6 ± 0.4 | -0.6 ± 0.1 |
| 8/23/68 | 0.5% ± 0.0 | -7.6 ± 0.1 | - |
| 9/6/68 | 0.5% ± 0.0 | -7.4 ± 0.2 | -0.8 ± 0.0 |
| 10/8/68 | 4.5% ± 0.5 | -8.9 ± 0.3 | -1.8 ± 0.0 |
| 10/22/68 | 9.5% ± 0.0 | -10.3 ± 0.3 | -2.8 ± 0.0 |
| 11/5/68 | - | - | -3.5 ± 0.0 |
| 11/11/68 | - | -11.2 ± 0.2 | - |
| 11/19/68 | 16.8% ± 1.6 | -10.7 ± 0.4 | - |
| 12/5/68 | 22.4% ± 0.1 | -11.5 ± 0.4 | - |

TABLE 2-2 (Cont'd):

| Date | Glycerol conc (gm%) | Supercooling Points (°C) | Freezing Points (°C) |
|---|---|---|---|
| 1/10/69 | 14.3% ± 2.3 | -10.6 ± 0.3 | -4.2 ± 0.0 |
| 1/22/69 | 22.5% ± 6.5 | -10.2 ± 0.2 | - |
| 2/14/69 | 10.5% ± 0.5 | -10.1 ± 0.2 | -4.3 ± 0.1 |
| 3/4/69 | 12.1% ± 1.5 | -11.1 ± 0.2 | -5.0 ± 0.1 |
| 3/17/69 | 19.8% ± 0.6 | -10.9 ± 0.2 | -4.9 ± 0.1 |
| 4/2/69 | 8.1% ± 0.7 | -10.7 ± 0.1 | -3.5 ± 0.1 |
| 4/21/69 | 3.0% | -7.7 ± 0.1 | -2.5 ± 0.1 |
| 5/6/69 | 0% | -6.4 ± 0.2 | -1.5 ± 0.1 |
| 5/27/69 | 0% | - | -1.3 ± 0.0 |
| 7/19/69 | 0% | -5.9 ± 0.2 | -1.3 ± 0.0 |
| 7/26/69 | 0% | -6.0 ± 0.2 | - |
| 9/9/69 | 0% | -7.9 ± 0.1 | -1.6 ± 0.0 |
| 9/18/69 | 0% | -7.1 ± 0.2 | - |
| 9/24/69 | 0% | -8.1 ± 0.3 | -2.0 ± 0.0 |
| 10/6/69 | 0% | -8.7 ± 0.2 | -2.6 ± 0.1 |
| 10/29/69 | 7.7% ± 0.4 | -10.7 ± 0.2 | -5.1 ± 0.0 |
| 11/28/68 | 10.7% ± 0.2 | -10.4 ± 0.2 | -5.3 ± 0.1 |
| 12/16/69 | 13.5% ± 0.5 | -11.0 ± 0.2 | -6.9 ± 0.1 |

Glycerol concentrations are mean ± S.D. Supercooling and freezing points are ± S.E.

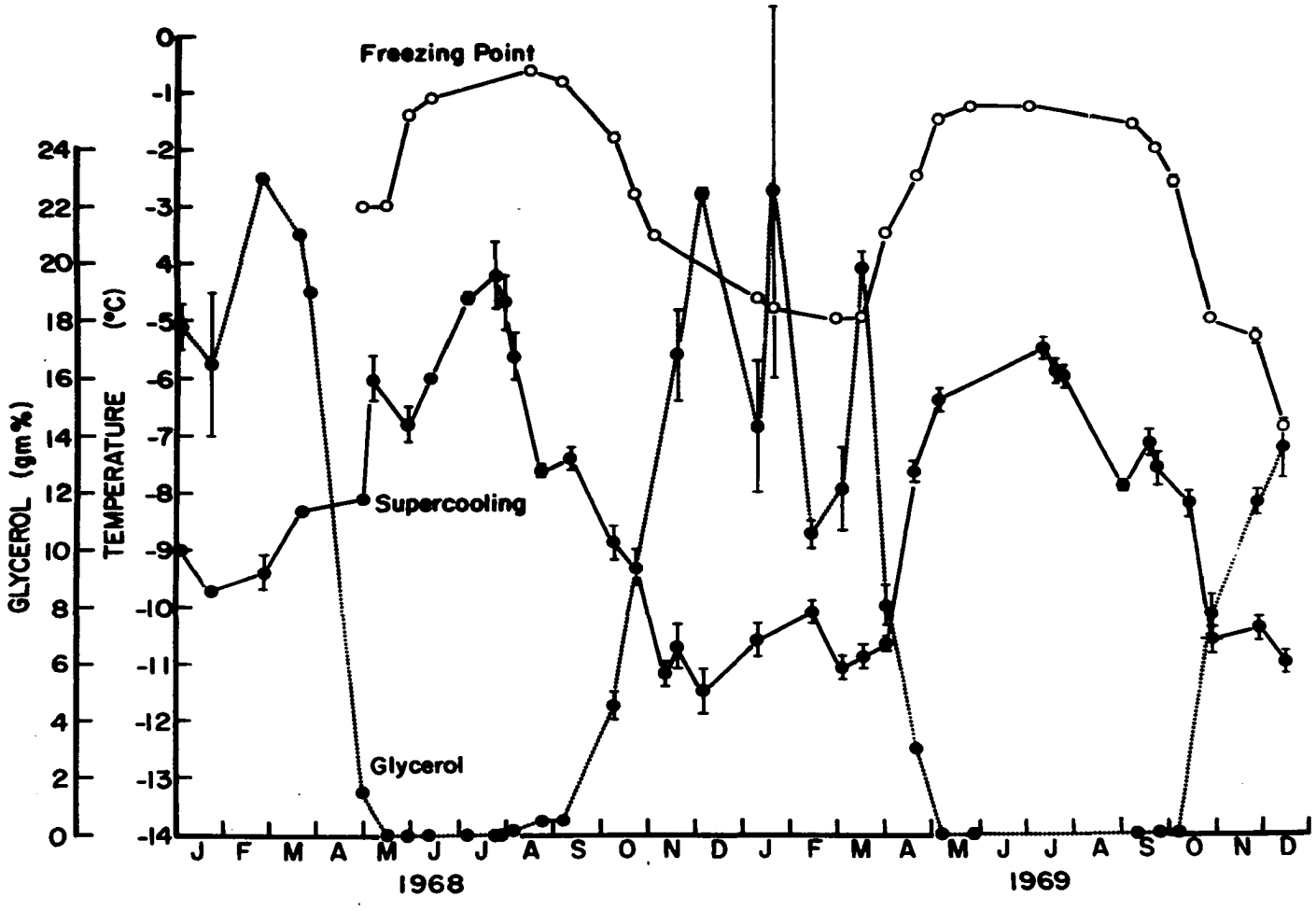


Figure 2-6: Illustrates the seasonal variations in hemolymph content and freezing points and whole body supercooling points in P. brevicornis.

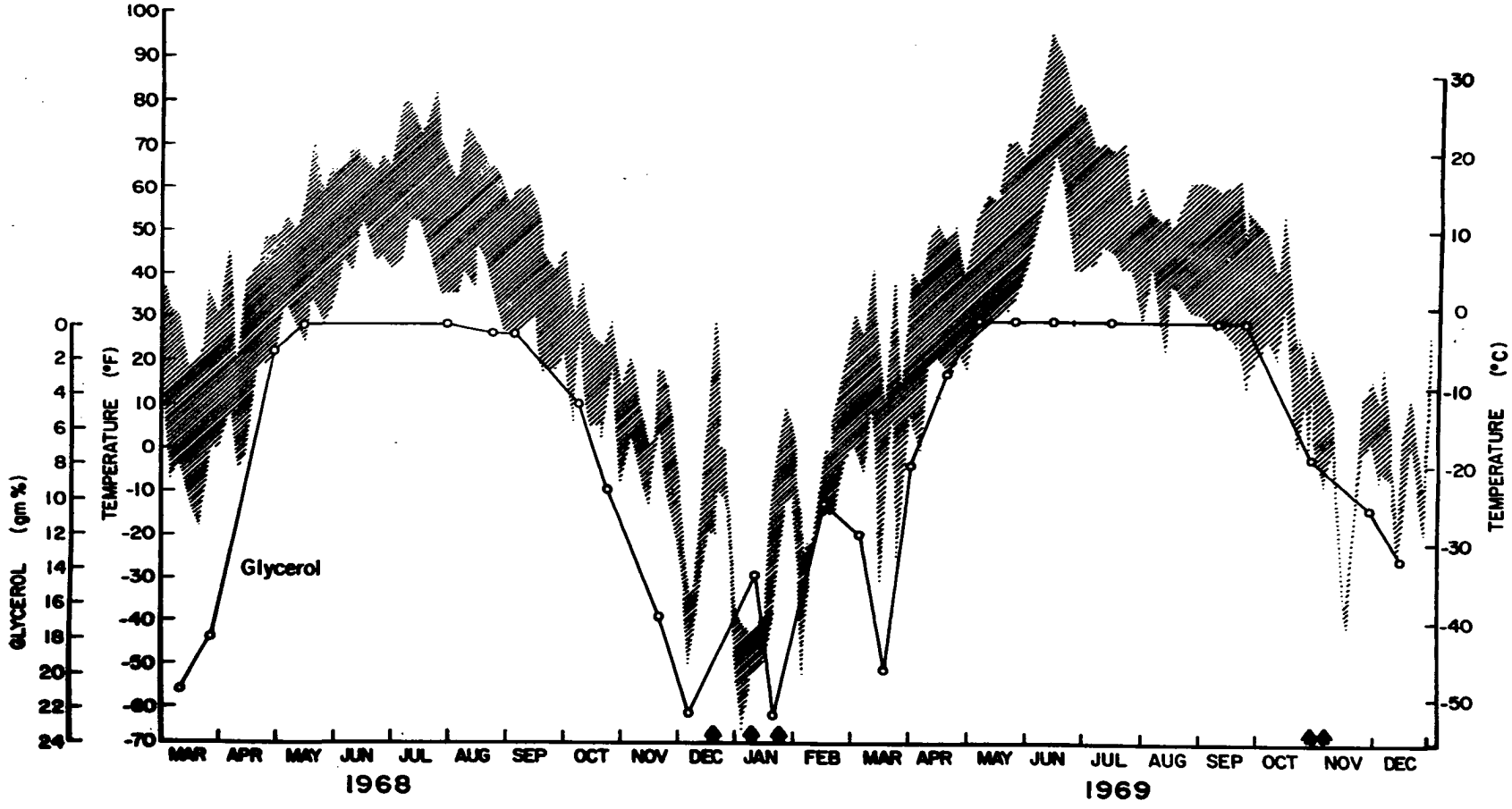


Figure 2-7: Seasonal variations in hemolymph glycerol content as related to fluctuations in daily high-low air temperatures.

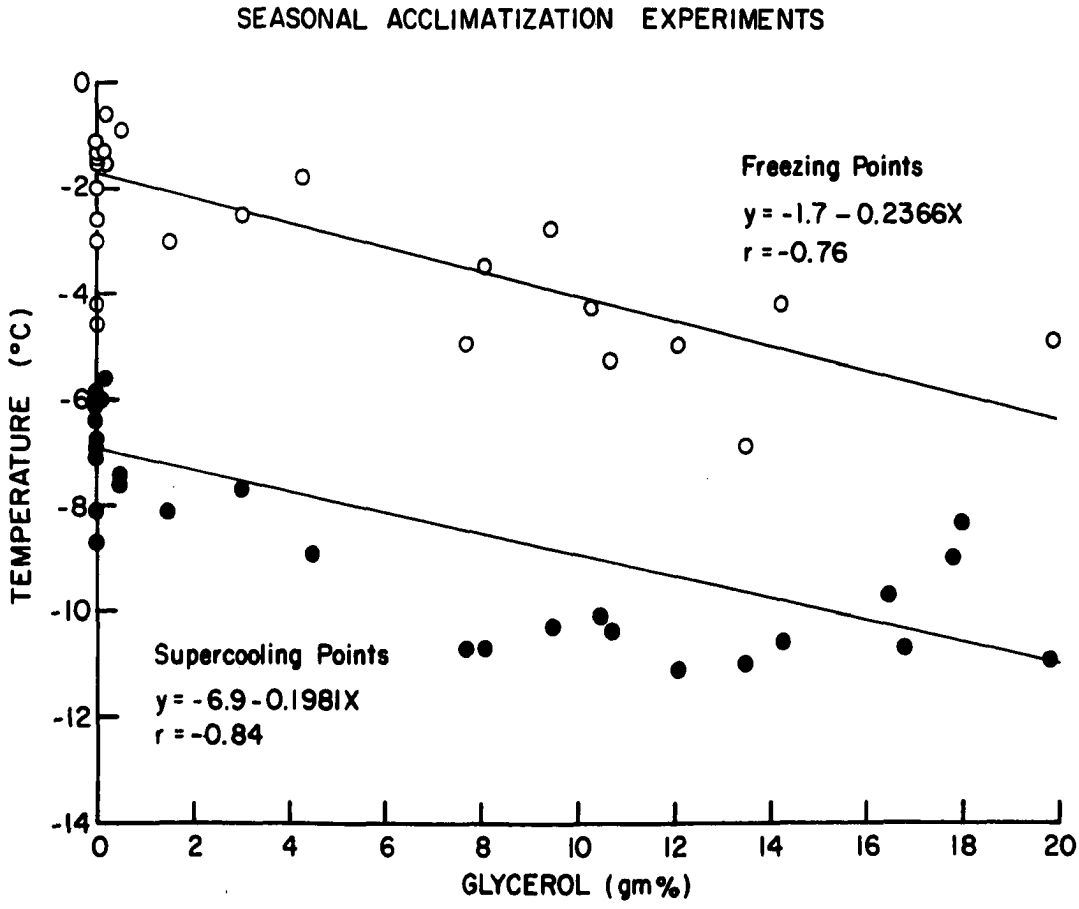


Figure 2-8: Linear regression lines of glycerol vs. freezing and supercooling points (r=correlation coefficient).

about 1°C throughout winter. Also, freezing points did not change as expected. A continued gradual depression occurred while glycerol levels diminished. Following a sharp elevation in glycerol content, freezing points decreased sharply.

Measurements of both hemolymph glucose and trehalose levels were made during periods of major glycerol variation in an attempt to define the probable carbohydrate origin of glycerol (Chino, 1957). Glucose was determined during late winter and early spring (1969). This period reflected changes in glycerol of upwards of 20gm%. However, no substantial amounts of glucose were indicated and no fluctuations occurred with respect to time and temperature exposure. Glucose levels were less than $10^{-6}$g/µl. That is, only a few milligrams of glucose were present within the adult insect's hemolymph. Trehalose levels were measured during fall and winter of 1969. While glycerol levels increased from 0 to 13.5gm%, significant variations in hemolymph trehalose levels were detected (Fig. 2-9). Due to the paucity of data, few conclusions may be drawn.

The direct relation between glycerol content and temperature is illustrated in Figure 2-7. Depicted are mean glycerol variations plotted against season and daily high-low ambient (air) temperatures. Again it can be seen that the initial stimulus to fall glycerol accumulation was first frost. An early frost in August, 1969, did not effectively stimulate the initial processes leading to glycerol synthesis. This was doubtless due to the lack of exposure of the insect to freezing temperatures. At this time the beetles were still on the forest floor. A

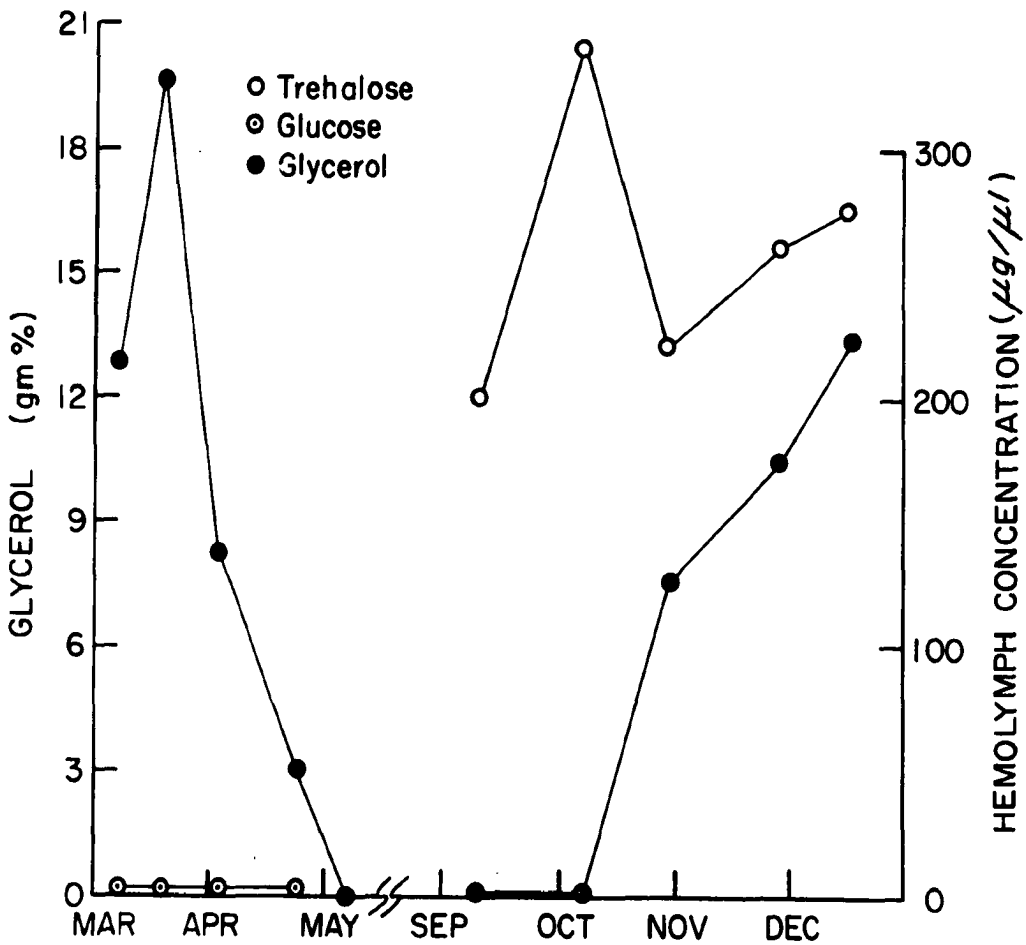


Figure 2-9: Seasonal variations in glucose, glycerol and trehalose concentrations in the hemolymph of P. brevicornis.

frost of such short duration would not be expected to reach and penetrate the mossy ground cover.

The glycerol fluctuations during December and later in March were directly related to temperature variations. While a plot of daily high-low temperatures do not directly coincide with habitat conditions, it is nontheless a close correlate especially when an insulative snow cover is absent. This was the case both in early December and March. During late December snow accumulated rapidly (about 2-3 feet). The early January dip in temperature was not reflected in the changed glycerol concentrations until later that month. This latter point is related to the relatively slow advance of the cold front through snow and the decayed stump (refer to Chapter 1).

Thermal Acclimation Experiments

Two series of experiments were conducted to determine temperature influence on rate changes in glycerol content along with concomitant changes in the physico-chemical parameters of supercooling and freezing. The first acclimation series illustrated the changes accompanying exposure of winter acclimatized beetles to spring (warm) temperatures.

Fourteen hundred specimens were collected in early February 1969 and placed in a freezer at -22°C ($\pm$ 2°C). This temperature approximated the mean habitat thermal state. The specimens were divided into six groups with one group each being placed at -14, -8, 0, +7, +14 and +23°C, respectively. Each group had specimens withdrawn at 24-hour intervals over three-day periods. Measurements of hemolymph glucose and glycerol

contents, freezing points and whole body supercooling points were made. These analyses were extended in the +7°C group to 5 days while in the +23°C group, determinations were made every 12 hours during the first two days. Figure 2-10 illustrates the time-rate of change as influenced by temperature in these groups. It should be noted that two collection samples were represented therefore the two zero points (Table 2-3).

The results were especially striking after acute exposure to above 0°C. Glycerol decreased from 19.8gm% to 2.4gm% after 24 hours at +23°C and to 0% following a 36-hour exposure. This represents a glycerol loss of 0.7gm% per hour. Supercooling and freezing points increased 0.09 and 0.04°C per hour, respectively. The loss of glycerol at +14, +7 and 0°C was relatively constant and approximated 0.3gm% per hour. Supercooling and freezing points increased at a greater rate when the temperatures were higher. For example, the supercooling points increased 0.01°C per hour at 0°C, and the rate increased to 0.07°C per hour at +7°C. Freezing points behaved similarly. At 0°C the rate of rise was 0.01°C per hour increasing to 0.05°C per hour at +7°C (Table 2-4).

Beyond this initial 24-hour period, the parameters of glycerol concentration and freezing and supercooling points continued to change. After 36 hours at +23°C, glycerol was lost and not regained. Freezing and supercooling over the same three-day period increased for 36 hours and then plateaued. This behavior was similiar at +14 and +7°C.

At 0°C and just sub-freezing temperatures, conditions were remarkably stable. The range of freezing and supercooling remained almost constant. One point, 0°C after three days digressed from this pattern. Mean

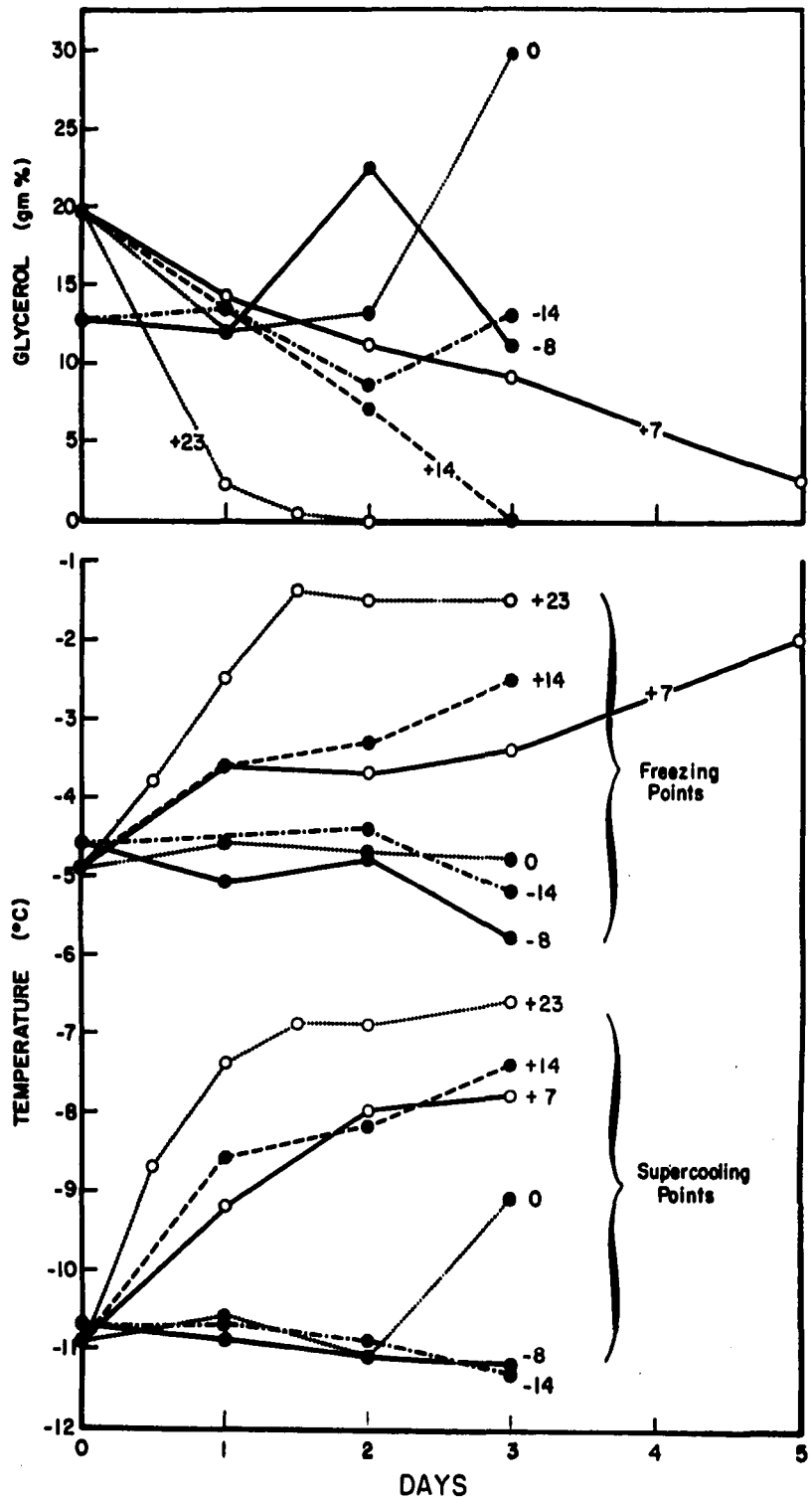


Figure 2-10: Variations in hemolymph glycerol content and freezing points and whole body supercooling points during warm (spring) acclimation in P. brevicornis.

TABLE 2-3

Changes in glycerol content, freezing points and supercooling points during spring (warm) acclimation.

| Acclimation temperature (°C) | Time (days) | Glycerol content (gm%) | Supercooling Pts. (°C) | Freezing Pts. (°C) | Glucose (gm%) |
|---|---|---|---|---|---|
| -22 | 0 | 12.8 | -10.7 | -4.6 | Trace |
| -14 | 1 | 13.5 | -10.7 | - | " |
| | 2 | 8.7 | -10.9 | -4.4 | " |
| | 3 | 13.0 | -11.3 | -5.2 | " |
| -8 | 1 | 11.8 | -10.9 | -5.1 | " |
| | 2 | 22.5 | -11.1 | -4.8 | " |
| | 3 | 14.1 | -11.2 | -5.8 | " |
| -22 | 0 | 19.8 | -10.9 | -4.9 | " |
| 0 | 1 | 12.0 | -10.6 | -4.6 | " |
| | 2 | 13.2 | -11.1 | -4.7 | " |
| | 3 | 29.6 | -9.1 | -4.8 | " |
| +7 | 1 | 14.3 | -9.2 | -3.6 | " |
| | 2 | 11.3 | -8.0 | -3.7 | " |
| | 3 | 9.1 | -7.8 | -3.4 | " |
| | 5 | 2.5 | - | -2.0 | " |
| +14 | 1 | 14.3 | -8.6 | -3.6 | " |
| | 2 | 7.1 | -8.2 | -3.3 | " |
| | 3 | 0 | -7.4 | -2.5 | " |
| +23 | 1/2 | - | -8.7 | -3.8 | " |
| | 1 | 2.4 | -7.4 | -2.5 | " |
| | 1-1/2 | 0.5 | -6.9 | -1.4 | " |
| | 2 | 0 | -6.9 | -1.5 | " |
| | 3 | 0 | -6.6 | -1.5 | " |

TABLE 2-4

Rate changes per hour[1] of glycerol content, supercooling points and freezing points at spring acclimation temperatures.

| Temperature (°C) | Glycerol Conc (gm%/hr.) | Supercooling Pts. (°C/hr.) | Freezing Pts. (°C/hr.) |
|---|---|---|---|
| -14 | SI[2] | 0 | - |
| -8 | SD[3] | SI | SI |
| 0 | 0.3 | 0.01 | 0.01 |
| +7 | 0.3 | 0.07 | 0.05 |
| +14 | 0.3 | 0.09 | 0.04 |
| +23 | 0.7 | 0.09 | 0.09 |

[1]Represents rate changes over the initial 24 hour period.

[2]Slight increase

[3]Slight decrease

supercooling points increased nearly 2°C between the second and third day. The glycerol picture, however, was not as straight-forward. At -14°C glycerol levels fluctuated slightly but generally remained stable. Unusual variations did occur, one during -14°C exposure and the other during -8°C exposure. The significance of these glycerol changes (data) may be questionable. The foregoing data are summarized in Tables 2-3 and 2-4.

The overall relationships between glycerol and freezing and supercooling points are illustrated in Figure 2-11. Freezing points were depressed 0.5°C per 4% glycerol increase while supercooling points were also lowered 0.5°C per 4% glycerol increase. The correlation coefficients are-0.75 (freezing points) and-0.59 (supercooling points).

Hemolymph glucose levels were measured in an attempt to correlate them with glycerol and possibly implicate glucose as a precursor (Chino, 1957 and Patton, 1963). Glucose levels were found to be low (less than $10^{-6}$g/µl) through the entire acclimation period without any apparent fluctuation.

Following two weeks of acclimation to +23°C, cold re-acclimation was attempted. Specimens were re-cooled stepwise at the same exposure temperatures. After five days no glycerol accumulation was observed at 0°C nor was there a depression of freezing and supercooling points. Below freezing exposure temperatures proved lethal.

Attempts to initiate glycerol synthesis were successful during cold acclimation of late summer beetles. Five thousand specimens were collected during late September 1969 and acclimated to +5°C for 1-3 weeks while in

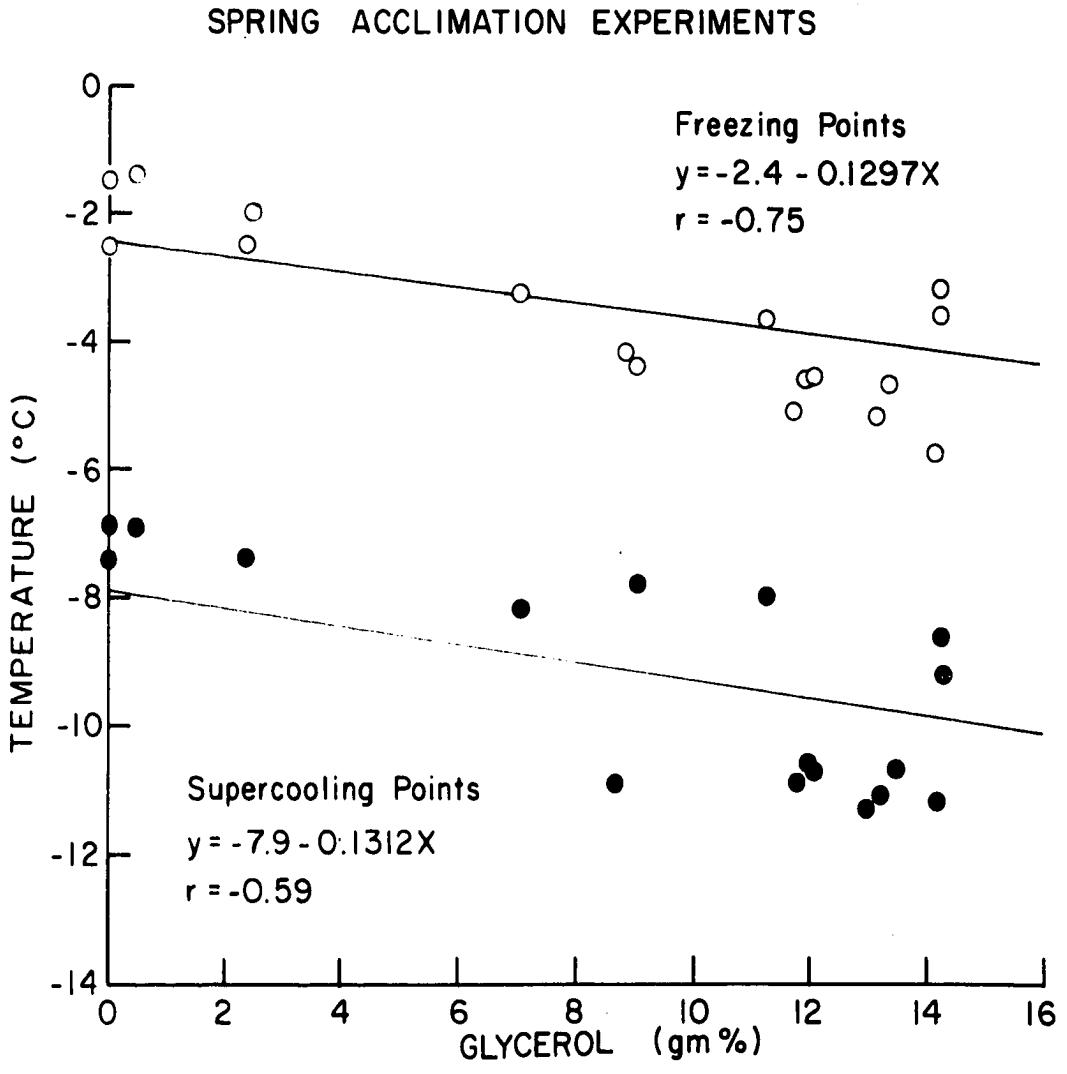


Figure 2-11: Linear regression lines of glycerol content vs. freezing and supercooling points during spring (warm) acclimation in P. brevicornis (r=correlation coefficient).

artificial stumps. Each "stump" contained 400-500 beetles. Specimens were then cooled stepwise, in 5°C increments, over 5-day intervals. After this period, the sample was divided into two groups, one group was maintained at the respective acclimation temperature while the remaining beetles were transferred to the next lower temperature. Table 2-5 is a schedule of this procedure.

Figure 2-12 illustrates the results of this 60-day experiment while Table 2-6 summarizes the data. In general, as acclimation temperatures decreased, hemolymph glycerol levels increased (Fig. 2-13) while freezing and supercooling points were depressed. At time zero (+5°C) no glycerol was present and freezing and supercooling points were relatively high, -2.0 and -7.3°C, respectively. Prolonged exposure to +5°C did not result in either the appearance of glycerol or significant changes in freezing or supercooling points (Fig. 2-12). Exposure to 0°C resulted in the appearance of glycerol after two days with a peak concentration evident after five days. A gradual oscillation in concentration followed. Freezing points mirrored changes in glycerol through the first three weeks with an unexpected drop during week four. Supercooling points lowered during week one and plateaued throughout the remaining weeks. At -5°C a similar variation in glycerol was observed, however, the initial peak advanced to day two with a following decrease to day seven. Beginning with week two through week four, a gradual increase in glycerol content was evident. Freezing and supercooling points reflected the changes in glycerol with the exception of the day two overshoot.

At the -10°C level of exposure, the insects were presumed "frozen"

TABLE 2-5

Schedule of fall (low temperature) acclimation experiments.

| 5000 Specimens | | | | | |
|---|---|---|---|---|---|
| Time 0: | +5°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| | to 0°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| Day 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 5 | | | | | |
| | to -5°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| Day 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 5 | | | | | |
| | to -10°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| Day 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 5 | | | | | |
| | to -15°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| Day 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 5 | | | | | |
| | to -20°C | 1 week | 2 weeks | 3 weeks | 4 weeks |
| Day 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 5 | | | | | |

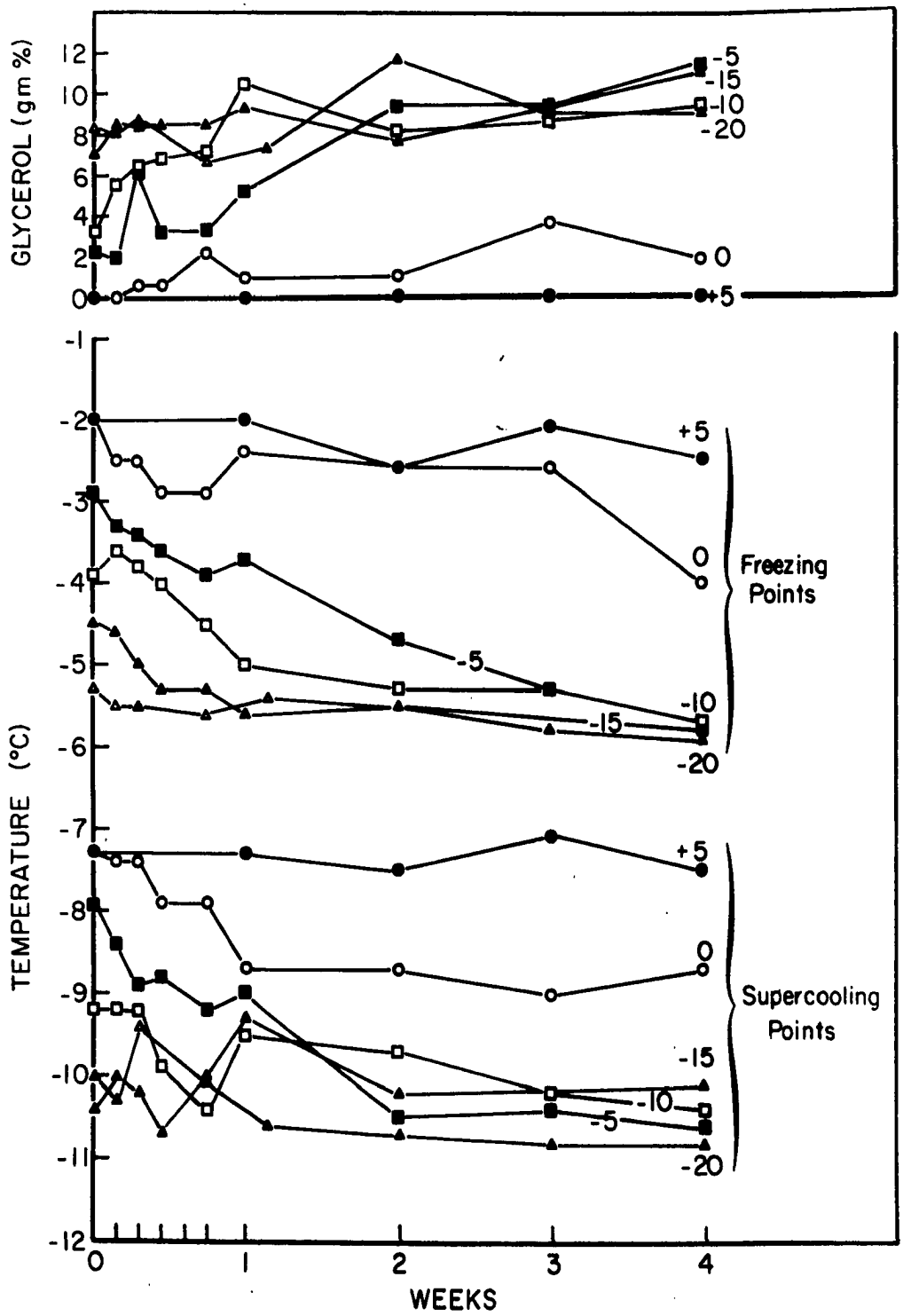


Figure 2-12: Variations in hemolymph glycerol content and freezing points and whole body supercooling points during fall (low temperature) acclimation.

TABLE 2-6

Changes in hemolymph glycerol, trehalose, and freezing points and whole body supercooling points during fall (low temperature) acclimation in *P. brevicornis*.

| Temperature Time | Hemolymph Glycerol (gm%) | Supercooling Pts. (°C) | Freezing Pts. (°C) | Trehalose (gm%) |
|---|---|---|---|---|
| +5°C 1 week | 0 | -7.3 ± 0.1 | -2.0 ± 0.1 | 3.4% |
| 2 week | 0 | -7.5 ± 0.1 | -2.6 | 4.0% |
| 3 week | 0 | -7.1 ± 0.0 | -2.1 ± 0.1 | 4.4% |
| 4 week | 0 | -7.5 ± 0.3 | -2.5 ± 0.0 | - |
| 0°C 1 day | 0 | -7.4 ± 0.1 | -2.5 ± 0.0 | 4.4% |
| 2 day | 0.5 ± 0.5 | -7.4 ± 0.1 | -2.5 ± 0.0 | 3.1% |
| 3 day | 0.5 ± 0.0 | -7.9 ± 0.1 | -2.9 ± 0.1 | 3.4% |
| 5 day | 2.1 | -7.9 ± 0.1 | -2.9 ± 0.0 | 5.4% |
| 1 week | 1.0 ± 0.0 | -8.7 ± 0.1 | -2.4 ± 0.0 | 3.0% |
| 2 week | 1.0 ± 0.0 | -8.7 ± 0.3 | -2.6 ± 0.2 | 5.0% |
| 3 week | 3.6 ± 0.1 | -9.0 ± 0.2 | -2.6 ± 0.1 | 5.7% |
| 4 week | 1.9 ± 0.4 | -8.7 ± 0.2 | -4.0 ± 0.0 | 3.2% |
| -5°C 1 day | 2.0 ± 0.0 | -8.4 ± 0.3 | -3.3 ± 0.1 | 3.4% |
| 2 day | 6.1 ± 0.0 | -8.9 ± 0.2 | -3.4 ± 0.1 | 4.4% |
| 3 day | 3.0 ± 1.0 | -8.8 ± 0.2 | -3.6 ± 0.0 | 2.7% |
| 5 day | 3.3 ± 0.3 | -9.2 ± 0.1 | -3.9 ± 0.0 | 3.0% |
| 1 week | 5.2 ± 0.1 | -9.0 ± 0.3 | -3.7 ± 0.0 | 3.3% |
| 2 week | 9.5 ± 1.1 | -10.5 ± 0.2 | -4.7 ± 0.0 | 5.2% |
| 3 week | 9.5 ± 1.0 | -10.4 ± 0.1 | -5.3 ± 0.1 | - |
| 4 week | 11.3 ± 0.5 | -10.6 ± 0.1 | -5.8 ± 0.1 | 2.7% |
| -10°C 1 day | 5.6 ± 0.0 | -9.2 ± 0.2 | -3.6 ± 0.1 | 1.9% |
| 2 day | 6.3 ± 0.5 | -9.2 ± 0.2 | -3.8 ± 0.0 | 1.2% |

TABLE 2-6 (cont'd):

| Temperature Time | Hemolymph Glycerol (gm%) | Supercooling Pts. (C°) | Freezing Pts. (°C) | Trehalose (gm%) |
|---|---|---|---|---|
| 3 day | 6.7 ± 0.2 | -9.9 ± 0.2 | -4.0 ± 0.2 | 1.2% |
| 5 day | 7.0 ± 0.0 | -10.4 ± 0.3 | -4.5 ± 0.0 | - |
| 1 week | 10.5 ± 0.3 | -9.5 ± 0.3 | -5.0 ± 0.0 | >0.5% |
| 2 week | 8.1 ± 0.1 | -9.7 ± 0.1 | -5.3 ± 0.0 | 5.4% |
| 3 week | 8.7 ± 1.7 | -10.2 ± 0.3 | -5.3 ± 0.1 | 2.4% |
| 4 week | 9.1 ± 0.1 | -10.3 ± 0.2 | -5.7 ± 0.0 | 1.7% |
| -15°C 1 day | 8.5 ± 0.5 | -10.0 ± 0.2 | -4.6 ± 0.0 | 4.3% |
| 2 day | 8.3 ± 0.3 | -10.2 ± 0.1 | -5.0 ± 0.1 | 4.0% |
| 3 day | 8.5 ± 0.8 | -10.7 ± 0.5 | -5.3 ± 0.1 | 5.2% |
| 5 day | 8.5 ± 1.1 | -10.0 ± 0.1 | -5.3 ± 0.1 | - |
| 1 week | 9.3 ± 0.2 | -9.3 ± 0.3 | -5.6 ± 0.0 | - |
| 2 week | 7.7 ± 0.9 | -10.2 ± 0.2 | -5.5 ± 0.0 | - |
| 3 week | - | - | - | - |
| 4 week | 11.1 ± 0.3 | -10.1 ± 0.2 | -5.8 ± 0.0 | 2.4% |
| -20°C 1 day | 8.2 ± 0.2 | -10.3 ± 0.1 | -5.5 ± 0.0 | 5.0% |
| 2 day | 8.8 ± 0.4 | -9.4 ± 0.2 | -5.5 ± 0.0 | 2.2% |
| 3 day | - | - | - | - |
| 5 day | 6.7 ± 0.7 | -10.1 ± 0.1 | -5.6 ± 0.1 | 2.1% |
| 8 day | 7.7 ± 0.7 | -10.6 ± 0.3 | -5.4 ± 0.1 | - |
| 2 week | 11.8 ± 0.2 | -10.7 ± 0.2 | -5.5 ± 0.1 | 3.6% |
| 3 week | 9.0 ± 0.3 | -10.8 ± 0.3 | -5.8 ± 0.0 | 2.6% |
| 4 week | 8.9 ± 0.1 | -10.8 ± 0.2 | -5.9 ± 0.0 | 2.8% |

± Standard error of mean

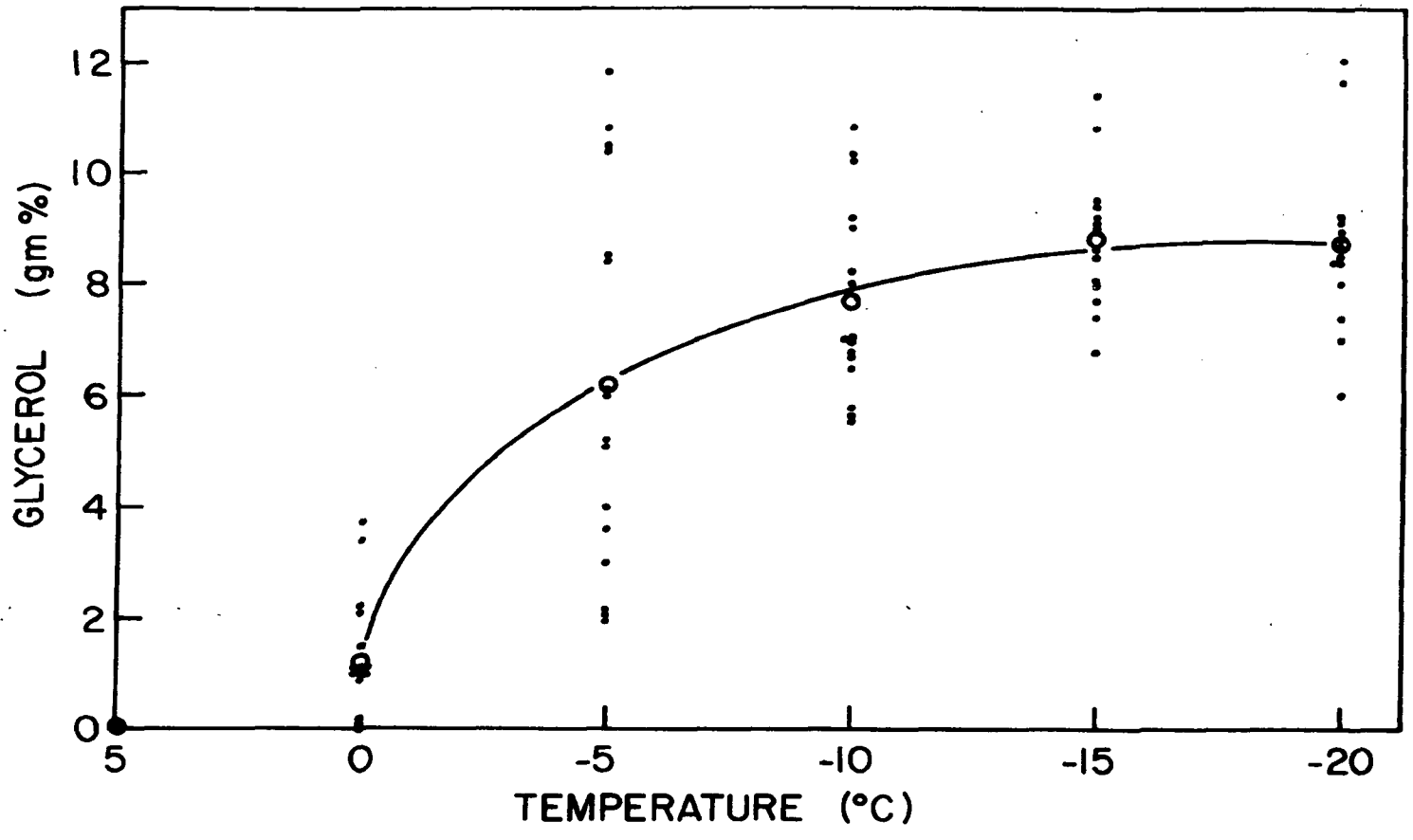


Figure 2-13: Plot of mean variations in hemolymph glycerol content at various acclimation temperatures (fall) in P. brevicornis (O= mean value at each temperature).

since the initial mean supercooling points were -9.2°C. During the apparent frozen state, glycerol accumulation continued over the first seven days at an approximate rate of 0.7gm% per day. Following this first week, glycerol levels decreased slightly and plateaued. Again freezing points mirrored changes in glycerol while supercooling points showed some variation during week one prior to conforming. Acclimation temperatures of -15 and -20°C resulted in generally similar curves. Glycerol remained high with some fluctuations evident. Freezing points were especially stable throughout the experimental sequence. Supercooling points on the other hand varied unexpectedly but generally remained low.

The direct correlation between depressions of the freezing and supercooling points and the increasing glycerol concentrations are evident in Figure 2-14. Correlation coefficients are -0.90 and -0.92 for freezing and supercooling points respectively.

In the course of this experiment hemolymph trehalose levels were measured in an attempt to again indicate the possible source of glycerol. Trehalose levels generally varied directly with changes in glycerol concentration. No direct correlation between trehalose and temperature were evident (Fig. 2-15). It should be recalled, however, that trehalose determinations were made on single, pooled samples at each acclimation temperature and therefore values may be questionable. Due to the relative accuracy of the analytical procedures and consistency of results, it is felt that inclusion of this data is worthwhile.

Initially, trehalose levels were at a high concentration ranging between 3.4 to 4.4gm% at +5°C. At 0°C the levels varied directly with those

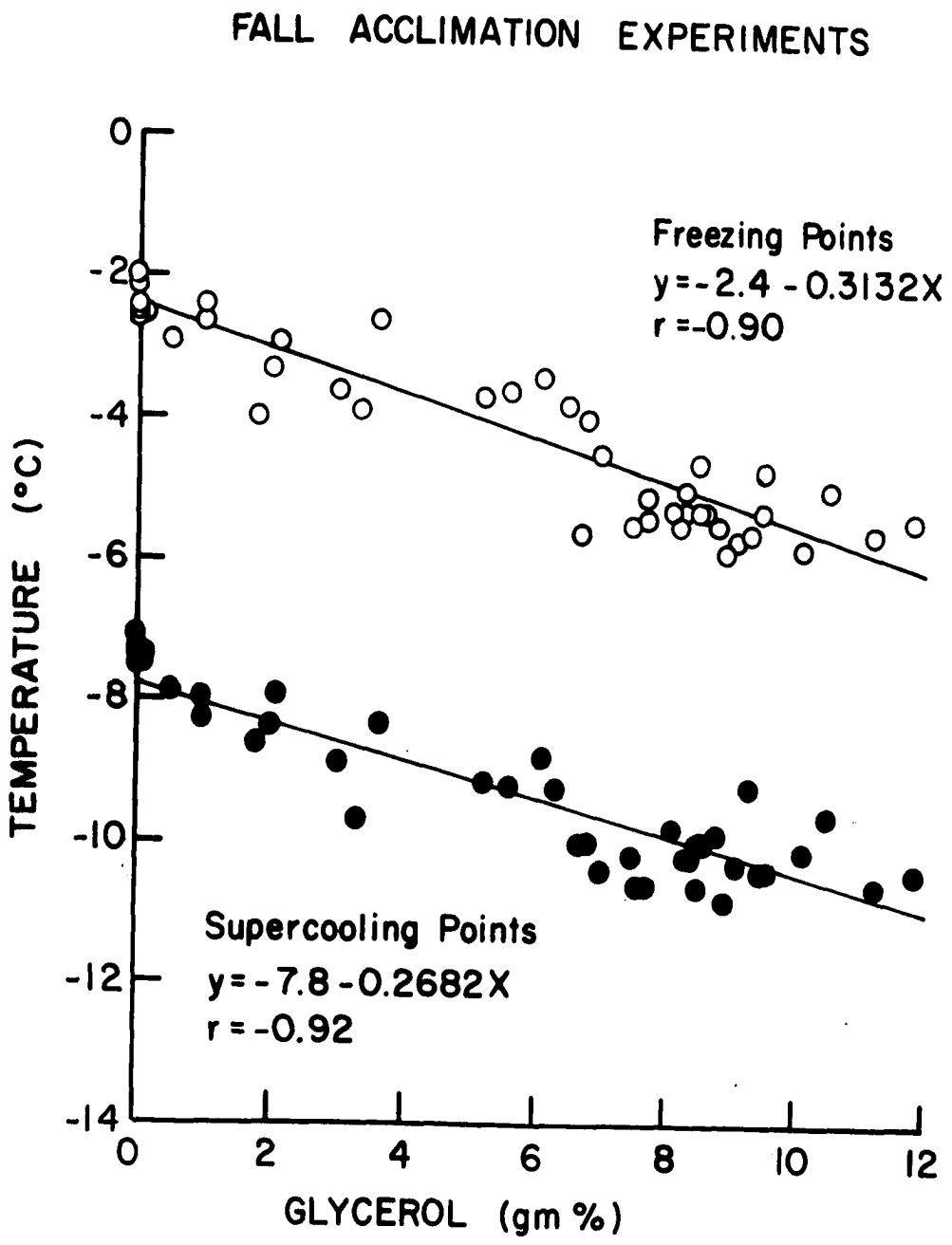


Figure 2-14: Linear regression lines of glycerol content vs. freezing and supercooling points during fall (low temperature) acclimation in P. brevicornis (r=correlation coefficient).

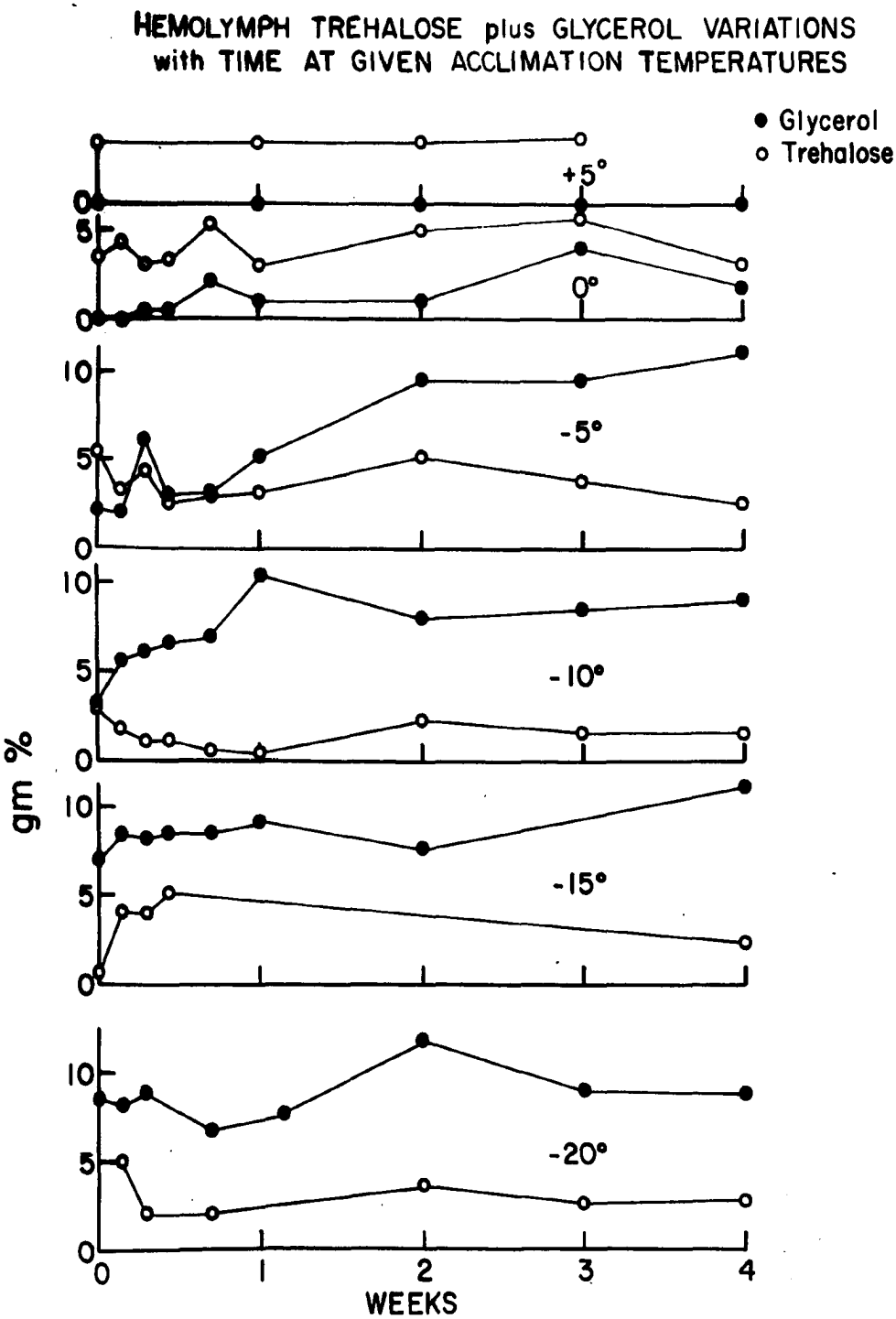


Figure 2-15

of glycerol excepting the first two days. This relationship held true during acclimation to -5, -15 and -20°C. At -10°C an exception was presented to this generalization; as glycerol levels increased over week one, trehalose levels decreased and it was not until the onset of week three that concentrations stabilized.

A number of points may be noted at this time regarding the above figure. First, at above freezing temperatures, +5, 0 and -5°C, trehalose levels generally varied with those of glycerol following the first few days of acclimation. During the first few days, trehalose decreased as glycerol increased. Second, at sub-freezing temperatures, -15 and -20°C, concimitant changes between glycerol and trehalose were evident throughout the experimental sequence. Finally, at-10°C, glycerol was accumulated at the most rapid rate. At this interim temperature in the region of the supercooling limit, trehalose decreased as glycerol increased during the first seven days. In the course of this week trehalose concentrations diminished from 3.0gm% to 0.5gm%. Since one molecule of trehalose may be considered to have the potential yield of 4 glycerol molecules (Fig. 2-16), it can be seen that the loss in this disaccharide could yield 10 moles glycerol. Glycerol concentrations increased from 3.3 to 10.5gm% over this week or a two fold increase (7.2gm) (Table 2-6). Assuming the direct source of glycerol to be carbohydrate and trehalose, the intermediate measured variations were more than sufficient to account for the observed glycerol increase, a 72% conversion of trehalose to glycerol.

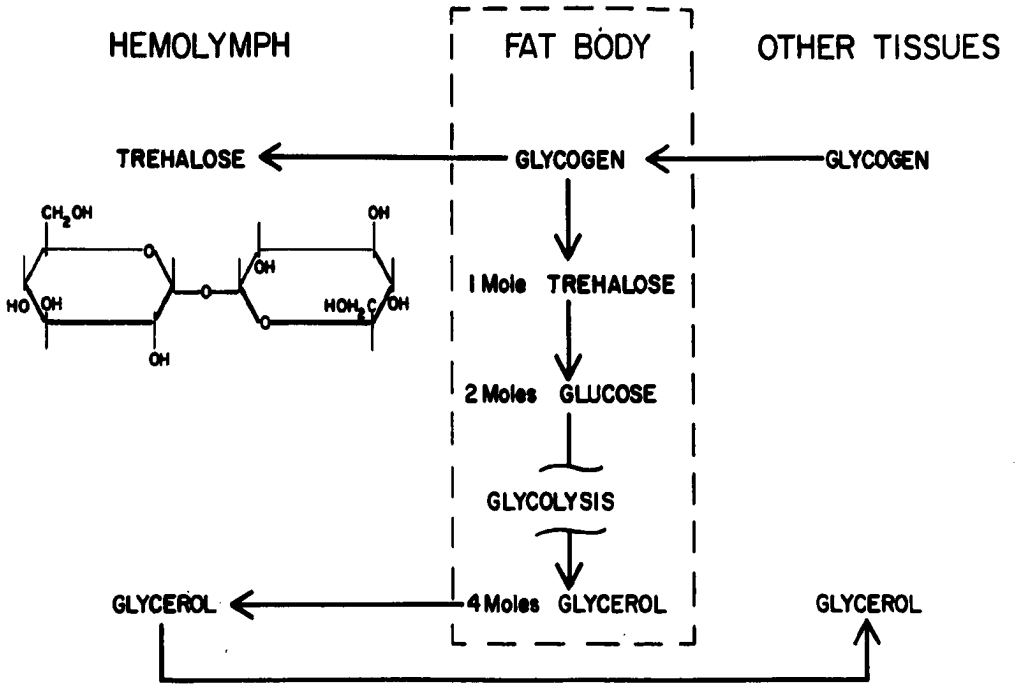


Figure 2-16: Illustrates the theoretical pathway for the conversion of glycogen to glycerol. Three body pools are indicated.

## DISCUSSION

It is evident from the foregoing data that glycerol accumulation and fluctuation was directly dependent upon ambient temperature. The stimulus to the initial synthesis was the first frost experienced within the microhabitat (Fig. 2-7). The peak December and March levels were directly related to peak low temperatures of the same period. The January peaks of glycerol and low ambient temperatures were offset. Doubtless this was due to the accumulation of an insulative snow cover during late December. That is, the stumps were no longer exposed as in early December but covered with 2-3 feet of snow. This insulating layer would tend to dampen the velocity of thermal fluctuation within the stump. The rapid temperature drop in late December (1968), about 55-60°F in five days, was not effective in cooling the well insulated microhabitat until mid-January. The rate of advance of the "frost front" was slow. Both the snow cover and the decayed stump had low thermal conductivities resulting in a slow heat loss.

Warmer temperatures during February resulted in a decrease in snow cover and greater exposure of the beetles to variations in ambient temperature. This was evident during mid-March. A sudden temperature drop resulted in a concomitant increase in glycerol concentration.

It is now apparent that variations in glycerol concentration directly effected the hemolymph freezing and whole body supercooling points. This relationship is statistically evident by the relatively high correlation

coefficients (Fig. 2-8, 2-12 and 2-15). Both the supercooling and freezing points were depressed on average of 0.9°C per 4gm% increase in glycerol. This parallel decrease was unexpected. Salt (1957) found that in the presence of glycerol, supercooling points were depressed further than freezing points for a given incremental rise in glycerol. However, Salt and most others to date have considered immature stages which were apparently passive to fluctuations in winter temperatures. That is, developmental processes (feeding, mobility, etc.) were thought to be absent during brief exposures to above freezing temperatures unless diapause was broken. P. brevicornis however does not behave in this manner. Kaufmann (1970) has shown that various processes are continued in spite of low temperatures and even in the "frozen state". She has observed continued egg development, food passage through the gut, waste excretion and variations in fat body size throughout winter. These changes were apparently not dependent upon warming (thawing)! The adaptative processes that have led to cold hardening in this beetle have surpassed those of the insects that only accumulate glycerol so that extended supercooling can take place (Somme, 1964).

There are a number of apparent inconsistencies present that are masked in a statistical evaluation of the data. These points are concerned with summer freezing and supercooling points and mid-winter freezing points. Referring to Figure 2-6, it can be seen that during early summer, freezing and supercooling points continued to elevate in spite of the lack of glycerol. One might comment that this data is contradictory. However, it should be realized that a number of changes are occurring

during this period that could drastically alter the biochemical make-up of the population. Certainly processes such as egg laying, mating, general maturation, changing metabolic rates, etc., could be expected to result in changes in hemolymph constituents, thereby contributing to the freezing point elevation. Increased total protein, carbohydrate (di- and polysaccharides), lipid and water content would be expected. Also, fatty acid saturation may occur (Buffington and Zar, 1968). Such saturation changes and increased concentration of large molecules would act to decrease the osmotic pressure of the hemolymph and result in elevated freezing points. Similiarly supercooling points would elevate. Accompanying changes in inorganic ions are unknown.

A second factor may contribute to the rising supercooling points. An increase in intestinal nucleators would be evident due to greater feeding activities. This in itself would greatly elevate supercooling points.

The inverse of these foregoing observations was evident during later summer. Prior to glycerol synthesis, freezing and supercooling points were depressed. The supercooling depression could possibly be attributed to reduced feeding and therefore fewer nucleators. Freezing point depression without any increase in glycerol may have been attributed to decreased high molecular weight solutes as a result of storage and a possible change in low molecular weight components. This latter change, however, was of such small amplitude, a few tenths of a degree, as compared to the supercooling point depression that it was insignificant toward contributing to cold hardening.

The relationship between mid-winter glycerol and freezing points presents a picture difficult to resolve. During this period glycerol concentrations were observed to fluctuate with changing temperature by as much as 10gm% but without any oscillations in freezing points. Throughout this period freezing points continued to be gradually depressed. These data are contrary to predicted and previously determined results. No speculations are presently offered concerning this observation. Throughout this same period supercooling points varied somewhat, about 1°C. This relative stability in supercooling range may be accounted for theoretically. Since P. brevicornis does not completely evacuate its gut prior to hibernation and since arousals were evident during warm spells with feeding occurring (Kaufmann, 1970), a high level of nucleators would be accrued (Salt, 1968). As glycerol levels were initially increased, a supercooling limit was reached at about 12-14gm% glycerol. Due to such factors as dehydration (Miller, 1969), solute concentration and resultant nucleator changes, further depression of supercooling points became impossible and stabilization resulted. This hypothesis is supported by the fact that during the February (1969) plunge in glycerol to levels of less than 12-14gm% was accompanied by supercooling point elevation. Also, following winter (late March) as glycerol levels plunged toward zero, supercooling points commenced to rapidly increase once glycerol concentrations were between 12-14gm%.

The results obtained in both acclimation series directly complemented the information obtained from acclimatization experiments. At warm acclimation temperatures the most striking changes were observed at above 0°C

ranges. Generally, as temperatures increased, glycerol content decreased and supercooling and freezing points were elevated. The higher the temperature, the greater the rate of change. At 0°C and sub-zero ranges, most parameters measured were stable. Glycerol concentrations remained stable with minor, expected fluctuations. There were, however, two unexplainable major variations, day two at -8°C and day three at 0°C. At these time-temperature regimes unusually high glycerol levels were encountered. Levels were so high in one case (day three at 0°C), that the validity of the two measurements may be questioned. This is especially evident when one considers the stability of freezing points over the same thermal ranges.

The most significant observation to be made from these graphs (Fig. 2-10) are the rapidity of rate changes with exposure to above zero temperatures. These changes were computed on a per hour basis in Table 2-4 and were greatest over the first 24 hour. At sub-zero temperatures most of the measurements were relatively stable.

While the warm acclimation experiments portrayed the events concurrent with the loss of the winter hardening adaptive processes, the fall acclimation series allowed for greater insights into the understanding of the physiological and biochemical events leading to this adaptation.

The initial stimulus to glycerol synthesis was exposure to 0°C for a period greater than 24 hours (Fig. 2-12). Continued glycerol accumulation was evident at subsequently lower temperatures. Also, at each acclimation step from 0°C to -10°C, an overshoot (two at 0°C) in glycerol concentration was evident. This peaking occurred on day five at 0°C, day two at -5°C,

and day seven at -10°C. Such responses are basic to physiological processes and may be considered protective (Bullock, 1957) Particularly, since glycerol synthesis is stimulated by continued decreasing temperatures, it would be of survival value to produce an excessive amount (anticipatory response) to such a stimulus in the event of a sudden, more rapid temperature decrease. However, it might be argued that the recovery (undershoot) would present a liability period.

Freezing points reflected the changes in glycerol content with few exceptions. These exceptions were: (1) when glycerol overshoots and oscillations were masked and (2) when occasional non-related changes were evident. The latter was the case at 0°C after four weeks. A rapid freezing point depression was observed while glycerol concentration decreased. Such contradictory measurements are doubtless a problem experienced while dealing with a population of individuals. No biological or chemically based reasoning can presently account for such a change. Supercooling points may also be correlated with changes in glycerol (Fig. 2-14), but again overshoots were masked. This in all probability was due to two factors: (1) the quantity of nucleators was gradually diminished due to reduced feeding activity and in turn (2) the quality of gut nucleators was modified in time (Salt, 1966, 1968 and 1969). This would account for the disproportional depression of the supercooling points at 0°C.

An interesting highlight to the modes of action of glycerol as a cryoprotectant (see Chapter 3) and its influence on cold hardiness might be found in an investigation leading to the isolation of the glycerol source. Two distinct possibilities exist. The first and most substantiated

implicates glycogen as the precursor (Chino, 1957 and Takehara, 1966). These studies, however, have left a number of unanswered questions and the data appear contradictory and incomplete.

Takehara (1966) indicated that 37mg/g of glycogen were utilized during the production of 25mg/g of glycerol. This would indicate an unrealistic efficiency of glycolysis (69%) and all but ignore intermediate considerations. This observation was in the fall. In the following spring, they observed a decrease in glycerol with a concomitant increase in glycogen and speculated as to the reversibility of this system. Their data indicates that 25mg/g glycerol was converted to 23mg/g glycogen! The basic fallacies in this conclusion are obvious. During the course of the studies, total body sugar was also determined and found to be constant! Such observations become untenable in light of their self-conflicting data. On the other hand, Chino (1957) has claimed to have found a quantitative relationship between glycerol and glycogen in the eggs of Bombyx. However, insufficient data was presented to formulate a conclusive argument.

A second potential source of glycerol might be lipids. The ready conversion of neutral fats (glycerides) to glycerol and their component fatty acids either by enzyme mediation or acid hydrolysis presents an attractive picture. As temperatures decrease, hemolymph pH would be expected to decrease (van den Berg, 1961 and 1969) during the onset of freezing and even prior to freezing due to reduced solubility of dibasic cations. This would effectively lower pH (slightly) and thereby potentiate lipase activity. While this latter point is speculative, it warrants further consideration in light of recent studies by Bruice

and Butler (1965). They found groups of unrelated organic reactions that were enhanced by the presence of ice. These were not enzyme catalyzed but directly related to increased hydrogen ion mobility through ice.

# CHAPTER 3

## THE PHYSICO-CHEMICAL ASPECTS OF GLYCEROL PROTECTION-

## MECHANISMS OF CRYOPROTECTION

## INTRODUCTION

The previous chapters have been concerned with certain aspects of the physiological, biochemical and to a lesser degree the ecological parameters resulting in gain or loss of cold hardiness in the insect, Pterostichus brevicornis. The functional cryoprotection afforded by glycerol has been indicated by this and other authors. Its natural and induced variations, influence on freezing and supercooling points and potential origins have been discussed. However, glycerol's modes of action have only been alluded to. It will be the object of this chapter to review and evaluate the current concepts of the nature of glycerol's protective action. This will entail a discussion of the related physical and chemical properties, its influence on solutions and biological fluids and the present schools of thought concerning the protective action of glycerol. A complete literature review and consideration of other cryoprotectants and their effects on a cross section of plant and animal tissue will not be attempted. An excellent review of this area may be found in Meryman (1966).

Glycerol possesses a number of interesting and unique properties that allow for varying degrees of protection in animal tissue during low temperature exposure. It is general knowledge that glycerol retards ice crystal formation. Interestingly, the freezing point of anhydrous glycerol is +18°C but due to its hydrophilic nature, extreme supercooling occurs. Glycerol is not only soluble in most electrolytes and non-electrolytes, but it also possesses solvent powers. The eutectic point for an aqueous glycerol solution (2:1) is -46.5°C.

For multiple phase systems, glycerol greatly lowers eutectic temperatures. The velocity of crystallization for pure glycerol at -43°C is less than $10^{-4}$mm per minute. This latter property is related to the increased viscosity of glycerol with decreasing temperature. For example, for each 20°C decrease in temperature below 0°C viscosity increases approximately one order of magnitude. Pure glycerol supercools to -83°C while a 95% aqueous solution supercools to -89°C. Upon freezing an aqueous glycerol solution, ice separates as a pure substance thereby effectively increasing molar concentrations of glycerol (Segur, 1953). Finally, the migration of certain inorganic ions (H+, $SO_4^{-2}$, $PO_4^{-2}$, etc.) is identical to or even greater than migration through water (Bruice and Butler, 1965).

Prior to discussing the manner in which glycerol acts to protect living tissues, a discussion of freezing damage is necessary. This topic leads to a web of confusion and contradiction, and it is here that various schools of thought regarding the nature of injury and protective action are found.

One of the earlier and obvious theories of freezing damage relates to the mechanical "damage" resulting from extra- and intracellular ice formation. Observations of the mechanical distortion of a tissue would appear to support this view. However, numerous references are available showing that many cell types, both vertebrate and invertebrate, withstand limited ice formation. Luyet and Gehenio (1940) concluded that there was no evidence of mechanical pressure or cell puncturing during freezing.

This latter possibility is apparently obviated by the fact that ice grows via accretion of water and not as it superficially appears, i.e. a traveling spear. Upon reaching the membrane, continued ice growth would be inhibited by the lipo-protein network. Crystal migration through the membrane is thought unlikely (Scholander, personal communication) due to the infinitesimal rate of ice growth through an "orifice" of 3-8 Å. This would effectively prevent intracellular freezing (Mazur, 1964) unless the cooling rate was very rapid (< 1000°C per minute). The above, however, are only theoretical conclusions. Salt (1969) believes that the membrane only presents a zone of reduced velocity of crystal growth and that innoculative freezing of intracellular compartments is inevitable. Levitt (1969) contends that intracellular ice occurs with time in plants. Reports of intracellular freezing, while infrequent, do occur in the literature (Salt, 1959; Losina-Losinsky, 1967). One is therefore left to choose between Mazur's mathematical predictions of the significance of cooling rates or Salt's theories on the influences of time and temperature on possible intracellular nucleation. Both theories have their attractions. This discussion will not attempt to choose between either worker but will discuss the role of glycerol as a cryoprotective agent in the presence of both extra- and intracellular ice.

Since the questions of (1) the nature and even the existance of mechanical damage and (2) whether extracellular ice results in intracellular innoculation and freezing are not resolved, it will be of value to speculate as to how glycerol could modify these factors in an

animal cell. The point of interest is at the supercooling point, the lowest temperature to which a liquid can be cooled without freezing. P. brevicornis gives a hint as to the manner in which glycerol protects against ice damage. Miller (1969) has shown that freezing in summer adults of this carabid was fatal. This correlates well with the findings of Baust and Miller (1970) that no glycerol was present during this season. Fall through spring, however, represented a period of glycerol presence and tolerance of body ice. The nature of the ice, intracellular and/or extracellular, was unknown. Variations in content due to glycerol fluctuations were not determined. Shinozaki (1962) has shown that ice content in frozen larvae varies with glycerol content and season.

Animals not possessing glycerol or other cryoprotectants in physiologically important concentrations would experience rapid ice spread. Initially, the extracellular fluids would increase in thermal conductivity allowing rapid heat loss. Accelerated ice growth would result, thus increasing total ice content. This ice would be unmodified in structure, i.e., needle like. As this ice front approached the cell membrane, the probability of fine spears accreting into and through pores would increase and in time intracellular freezing might be expected with resultant cell death.

Such a picture is dramatically modified by the influence of glycerol. Figure 3-1 represents the ice crystal structure of water and various aqueous glycerol solutions. Photomicrographs were taken on a freezing stage regulated to -15°C $\pm$ 0.5°C after six minutes of

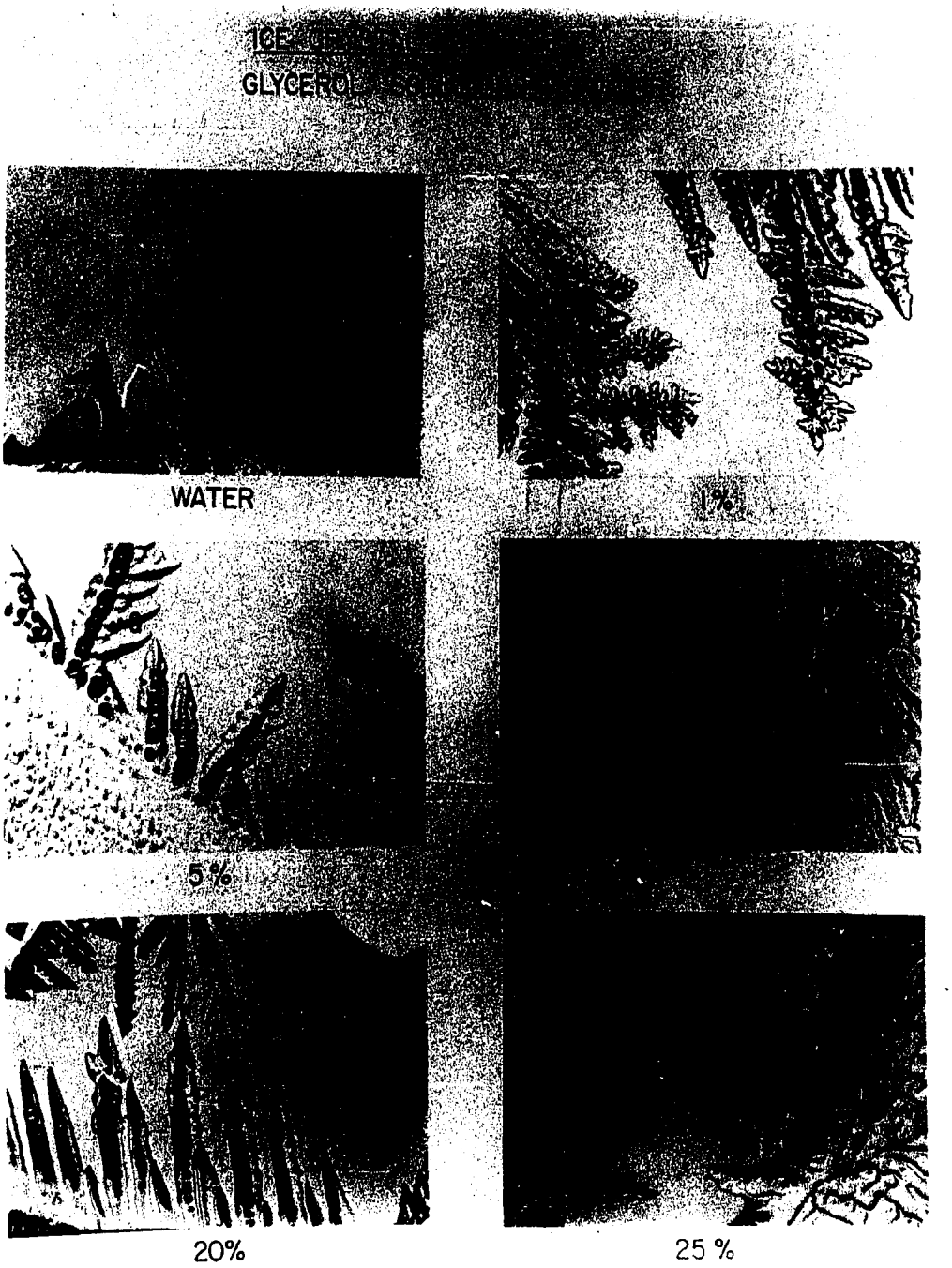


Figure 3-1: Photomicrographs of ice crystal structure of pure water (W) as compared to ice crystal structure in the presence of various glycerol solutions (G) magnification 100 X.

growth. Magnification was 100 diameters. Gross crystal structure changes were evident with increasing glycerol concentrations [each photograph contains ice crystals originating from either pure water (W) or an aqueous glycerol (G) solution]. The needle-like structure was blunted as glycerol levels increased. Total ice growth (content) also diminished as glycerol increased. Contributing to these changes were the resultant increase in viscosity and stable thermal conductivities. The latter would be expected to increase with decreasing temperature but is counterbalanced by the increased solute concentration (Doebbler, 1966). Further consequences to be predicted by diminished ice content are (1) inhibition of intracellular ice formation, (2) little or no membrane "puncture", and (3) inhibition of membrane denaturation.

Other hypotheses of the mechanisms of injury are concerned not with ice itself but with the resulting dehydration and increased solute concentration. Two basic schools of thought are evident with respect to these phenomena. The first is concerned with the loss of protein surface water. Advocates speculate that bound water is either lost from the protein surface, causing denaturation (Karow and Webb, 1965), or that cellular dehydration leads to a reduction in the distance between individual proteins. Such distance reduction would foster the formation of abnormal disulfide bonds. This rearrangement occurs either through oxidation of sulfhydryl bonds or disulfide interchange (Levitt, 1962).

Glycerol may be expected to prevent or at least inhibit the above

actions by retaining water in solution thereby retarding dehydration. Also, this effect in conjunction with that of the solvent nature of glycerol would act to maintain protein integrity (spacing).

Lovelock (1953) proposed one of the more workable hypotheses concerning cell damage and glycerol's protective role. As water freezes out of an extracellular aqueous solution, electrolyte levels were postulated to increase proportionally until lethal levels were reached. Lovelock found that for red blood cells this level was 0.8M sodium chloride independent of temperature. Similarly, as the electrolyte concentrations increased extracellularly, intracellular water diffused out in response to the newly imposed osmotic gradient. Glycerol would be expected to reduce electrolyte concentrations at any given low temperature via reduced ice content and therefore less dehydration. That is, the mole fraction of salt in the liquid phase (aqueous glycerol solution) in equilibrium with solid ice would be less than the corresponding mole fraction of salt in the liquid phase of an aqueous salt solution. This action may be termed electrolyte buffering (Farrant, 1969). A further effect of glycerol would be to lower eutectic points. This would prevent precipitation of salts and thereby avoid protein denaturation.

Meryman (1969) has proposed yet another attractive theory of freezing damage. Based upon data obtained from both red blood cells and mollusks, he has concluded that electrolyte concentration is not as important as the "minimum critical cell volume". As freezing progresses and cell water is lost, cells shrink. Shrinkage is thought

to be tolerable until a given minimum size is attained. At this point membrane rupture would occur.

Glycerol would function by preventing volume decrease. Since it is presumed to penetrate freely through the cell membrane, glycerol would be osmotically indifferent. This would allow for large glycerol concentrations without altering cell volume. Also, the hydrophilic nature of glycerol would aid in retaining water, solubilizing salts and decreasing ice content. Evidence supporting this theory is attractive but not conclusive.

A final theory suggests that damage is not due to initial freezing in all animals but is related to a phenomenon peculiar to rewarming or constant low temperatures. This phenomenon is termed recrystallization. When solutions are cooled to a specific low temperature ice structure may be observed to change with time (dependent on cooling rate and solute concentrations). This change, migratory recrystallization (Luyet, 1966), is thought due to larger crystals growing at the expense of small crystals. Similar observations have been made during rewarming but the mechanism is unclear. This form of recrystallization differs from irruptive recrystallization in that ultra-rapid cooling rates are not prerequisite. It is therefore applicable to the insect situation.

Migratory recrystallization is thought to increase osmotic stress by rapidly elevating ice and electrolyte contents and decreasing water content. Also, possible mechanical stress becomes an important factor. Glycerol would act in a number of ways to offset these lethal factors.

Luyet (1960) has found that cryoprotectants such as glycerol greatly decreased the recrystallization temperature. As a matter of fact, with glycerol, an organism would not naturally experience the low temperatures necessary for this form of recrystallization (-70 to -90°C).

The complexities of interaction of the above mechanisms of freezing damage along with the counter interactions of glycerol become overwhelming. Asahina (1966) has reacted to this problem but putting forth yet another theory of freezing damage. The discussion, however, was restricted to insects. He proposed the "site of freezing" concept which basically stated that extracellular ice was generally tolerable but that intracellular ice would be fatal! The basis of this theory was obscure in light of numerous studies (Salt, 1959, and 1962; Losina-Losinsky, 1956; etc.) and therefore must be seriously questioned.

## DISCUSSION

An integrated illustration of the results of freezing in animal tissue with and without glycerol is presented in Figure 3-2. Construction of this figure is based upon the previous discussion and represents the accumulated efforts of this and numerous other studies. One basic assumption was made, cooling rates of a few degrees per minute or less would be experienced by the animal tissue in question. Phenomena such as vitrification, irruptive recrystallization, etc., are not applicable. Slow cooling conditions were the norm of the insect studied. Maximum potential cooling rates experienced within the microhabitat must be

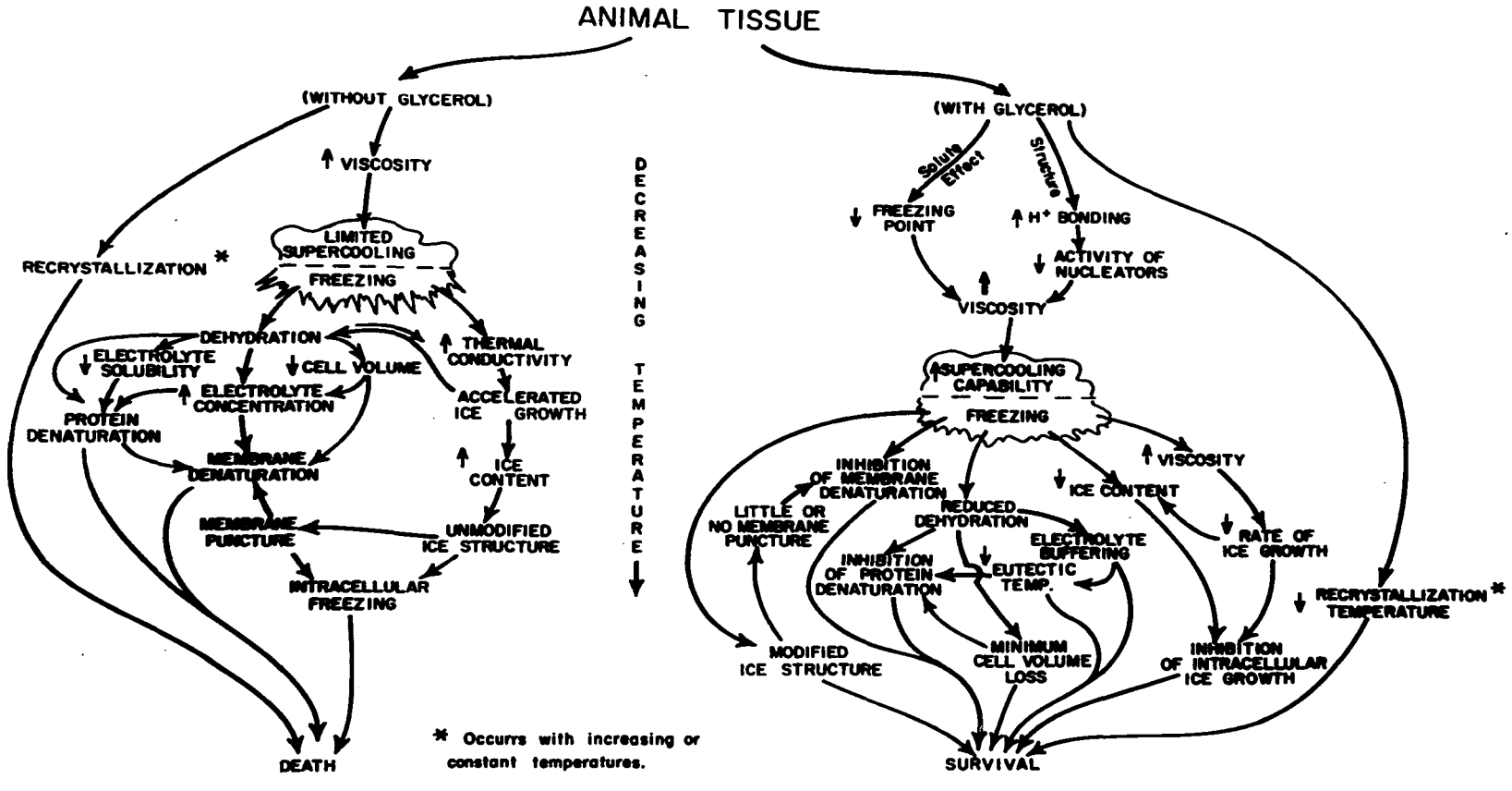


Figure 3-2: Schematic representation of the mechanisms of action of freezing damage and cryoprotection of glycerol in animal tissue.

considered slower than the maximum daily (short term) decrease in ambient temperature ($\sim$ 0.5°C/hour/day, see Chapter 1) recorded over a two-year period.

Critical review of Figure 3-2 will reveal the intricacies of freezing damage and glycerol interaction. Let us first refer to the glycerol-free tissue for the effects of temperature decrease. Fluid viscosity, both intra- and extracellularily, would increase. Some indications of the order of magnitude to be expected are evident if one looks at viscosity changes with temperature in non-physiological solutions. As water supercools from 0 to -10°C, viscosity increases approximately 0.1 centipoise/°C (Weast, et al., 1964). However, viscosity of pure glycerol increased 3520 centipoises for the same incremental temperature decrease (Dorsey, 1940). This represents a 35,000 X rate of viscosity increase. The absolute viscosity changes in hemolymph are certainly less pronounced but nonetheless important to the system. For example, a 20% aqueous glycerol solution is 2 X as viscous as water at 0°C. More extensive data on rate of change of various glycerol solutions are not available.

As temperature further decreases in the glycerol-free tissue, supercooling will result. The range of supercooling will be small but dependent on availability and quality of nucleating sites. Spontaneous freezing occurs next with two resulting changes: dehydration and a gradual increase in thermal conductivity. The resulting damage from dehydration may take one or more of five forms. First, electrolyte concentrations elevate acting to denature both cellular proteins and the

cell membrane. Second, electrolyte solubility will decrease and the precipitation of certain ions (dibasic) is probable. Similarly, cell volume would be expected to decrease as water is lost. Both of these latter occurrences could lead to membrane denaturation and death. With the increasing thermal conductivity (somewhat buffered by the increased solutes), further dehydration due to accelerated ice growth would occur. As the ice grows, the total ice content increases and the crystal structure will be needle-like (unmodified) contributing to membrane "puncture" (accretive ice growth through cell pores). Innoculative intracellular freezing will result. These three factors, intracellular freezing, membrane and protein denaturation may independently or in conjunction lead to irreversible damage. A fourth factor, migratory recrystallization, may also contribute a damaging effect at warming or static temperatures as high as -15°C.

The beneficial effects of glycerol on this tissue system (P. brevicornis) may be considered next. Two influences at above freezing temperatures are evident. The first is the solute effect. An increased solute concentration will act to depress the freezing point thereby increasing viscosity and prolonging the liquid state. The second effect is dependent on structural characteristics. Hydrogen bonding increases greatly partly due to glycerol's hydrophilic nature. This results in decreased action of nucleators through competitive action of water and glycerol on the nucleating site. Glycerol may also bond water thus gaining a further advantage over the nucleator. This action also retards freezing and allows for increased viscosity thereby contributing

to further supercooling. The extent of supercooling point decrease will ultimately depend on glycerol concentration and nucleator state. Absolute depression is usually not greater than 20°C in insects with ∿ 10°C being the norm.

Once freezing is initiated, a number of interrelated changes occur that contribute to cryoprotection. Viscosity continues to increase in the unfrozen liquid slowing the rate of ice growth. The diminished growth leads to decreased total ice content and both in turn act to inhibit the formation of intracellular ice. Dehydration is also reduced due to hydrogen bonding of water to glycerol. Limited dehydration acts to buffer electrolyte build-up as compared to glycerol-free tissue. Eutectic points are greatly reduced to levels below those which the insect will naturally encounter. The retention of extracellular water reduces the osmotic gradient between intra- and extracellular compartments allowing only a minimum loss of cell volume.

These effects (decreased eutectic temperatures, maintenance of cell volume and minimal dehydration) act to inhibit protein denaturation, i.e., loss of protein bound water and formation of disulfide bonds. Maintenance of protein integrity contributes to inhibition of membrane denaturation. Glycerol further acts to modify ice structure via crystal blunting. This will reduce membrane "puncture", along with reducing mechanical stress, and will help to preserve the membrane structure. Finally, recrystallization temperatures will be lowered to levels well below natural exposure. On rewarming, migratory recrystallization will not occur.

## SUMMARY

From the discussion presented in the preceding pages, it is obvious that an exact understanding of the mechanisms of action of glycerol as a cryoprotectant is either yet to be discovered or obscured within the maze of interactions. New break-throughs will certainly come with refinement in instrumentation and techniques. However, it seems self-evident in light of the research to date, that new concepts will out of necessity be based on the foundations so far established. One of the problems facing the cryobiologist is that the systems generally studied are somewhat "artificial". In vitro studies of red blood cells, spermatozoa, mammalian organs, etc., while contributing to the clinician's knowledge of cryosurgery and cryopreservation, are hindered by the fact that the most efficient cryoprotectants, DMSO and glycerol, are lethal in concentrations sufficiently high enough to afford freezing protection (Smith, 1961).

It is apparent that a multi-cellular system (organism) that naturally possesses glycerol in high, non-lethal concentrations, which can maintain activity while levels are high, which can survive freezing and, finally, which can regulate cryoprotectant levels in the presence of changing environmental conditions, should be a good focus for future studies. A group of carabid beetles (Pterostichus subgenus Cryobius) display these characteristics and warrant continued study.

# CHAPTER 4

## TEMPERATURE INDUCED NEURAL ADAPTATIONS

# INTRODUCTION

In the preceding chapters a number of adaptative processes, both behavioral and physiological, have been described in relationship to winter survival in P. brevicornis. In light of the observed coordinated activities, walking, etc., at sub-zero freezing temperatures, it became important and necessary to consider a neurophysiological correlate to overwintering. If representation of a sustained activity at sub-zero temperatures could be described, it would be of profound survival value.

The lack of information related to neural function at low temperatures in invertebrates and particularly insects is striking (Bullock and Horridge, 1965). Recent studies have almost exclusively concerned themselves with either the analysis of peripheral thermoreceptor activity (Loftus, 1968 and Dethier and Schoonhoven, 1968) or with the effects of temperature on rate changes in central (i.e. ganglionic) discharge patterns (Kerkut and Taylor, 1956 and 1957). A third trend has also evolved which considers measurements of threshold (Boistel, 1957 and Bernard et al., 1961) or excitability (Bernard et al., 1965). In general, however, these studies have centered around organisms not normally exposed to temperatures cooler than laboratory conditions and certainly not below temperatures much above freezing ($\sim$ +5 to 10°C). Such approaches have precluded understanding of the mechanisms allowing for continued activity in insects subject to extreme annual and daily cold exposures.

A number of responses have been described in an attempt to explain continued activity in insects at low temperatures. These studies have revolved around both behavioral and limited metabolic thermoregulatory

considerations, but again do not consider low temperature forms. For example, locust orientation to sunlight resulting in elevated body temperature prerequisite to flight was described by Frankel (1924). Vielmetter (1958) has discussed the adjustment of wing position in butterflies so as to regulate absorption of radiation. Recently Adams and Heath (1964) have discussed "shivering" in the sphinx moth as a method of maintaining an elevated body temperature in lieu of "peak" activity cycles.

Arctic and near-arctic insects are faced with a problem in that the above mechanisms are not suitable except for short periods during mid-summer. The maintenance of elevated core temperatures in such poorly insulated insects in the presence of extreme thermal gradients would be metabolically unfeasible. This then would present a limited breeding and winter preparatory period, which for most insects would be too short. This problem is compounded by the fact that many northern insects "freeze" completely (Scholander et al., 1953, Miller, 1969 and Kaufmann, unpublished). Such observations, until recently, have led to the erroneous conclusion that all insects are dormant at low temperature.

## METHODS AND MATERIALS

Experiments were conducted in a darkened cooling chamber. Intact organisms were mounted upside down and rigidly secured in beeswax. The recording electrode (0.001" Pt wire) was inserted into the trochanter of the hind thoracic leg and advanced to a position just distal to the coxal juncture. The actual recording surface of the electrode was calculated to be $10^{-2}mm^2$. An indifferent electrode was inserted beneath the

abdominal cuticle. Efferent potentials were amplified on a Grass P5 AC pre-amplifier operated in the single ended mode. The amplified potentials were monitored on a Tektronix 565 oscilloscope and simultaneously recorded on tape and electronically counted (Fig. 4-1 and 4-2).

Insect temperatures were determined on a recording potentiometer connected to a 36 gauge copper constantan thermocouple placed beneath the abdominal cuticle.

Specific neuromuscular junctional inhibitors and transmitters were applied directly to the surface and/or injected into the muscle of the trochanter. The effects of these compounds on efferent discharge were noted in an attempt to elucidate the origin of the discharge activity, i.e. motor fiber spike versus muscle potential.

Acclimatization studies utilized outdoor subjects while acclimated specimens were stored in environmentally regulated cold rooms and freezers.

## RESULTS

Specimens were cooled until efferent spike activity was no longer evident. In winter beetles this occurred following supercooling (freezing) whereas motor discharge terminated prior to the initiation of supercooling in the summer beetle.

Discharge patterns differed quite markedly between these two conditions. This difference is illustrated in Fig. 4-3. The summer frequency pattern approximates an exponential drop and is indicative

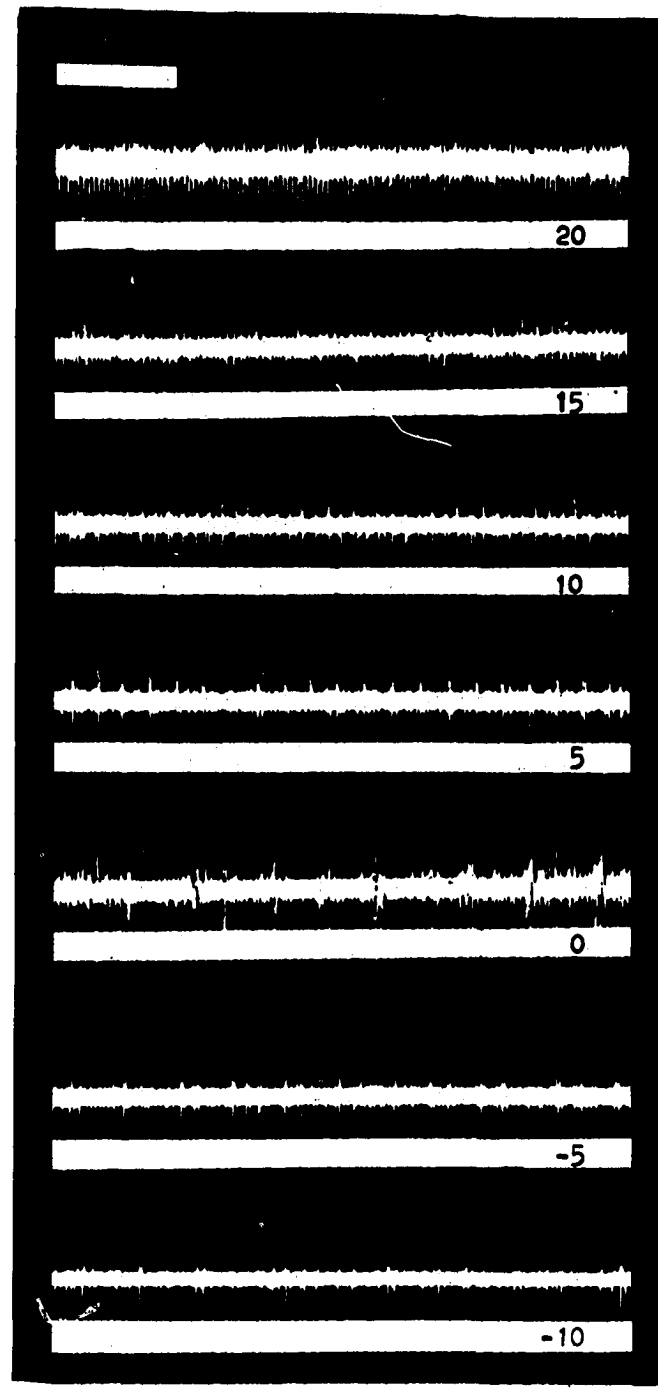


Figure 4-1. Illustrates a sample efferent spike discharge over various temperatures. Along with varying frequencies, spike durations, amplitudes and polarities were observed to change. Lower trace is calibration pulse of 30uV while line in upper illustration represents 1 sec.

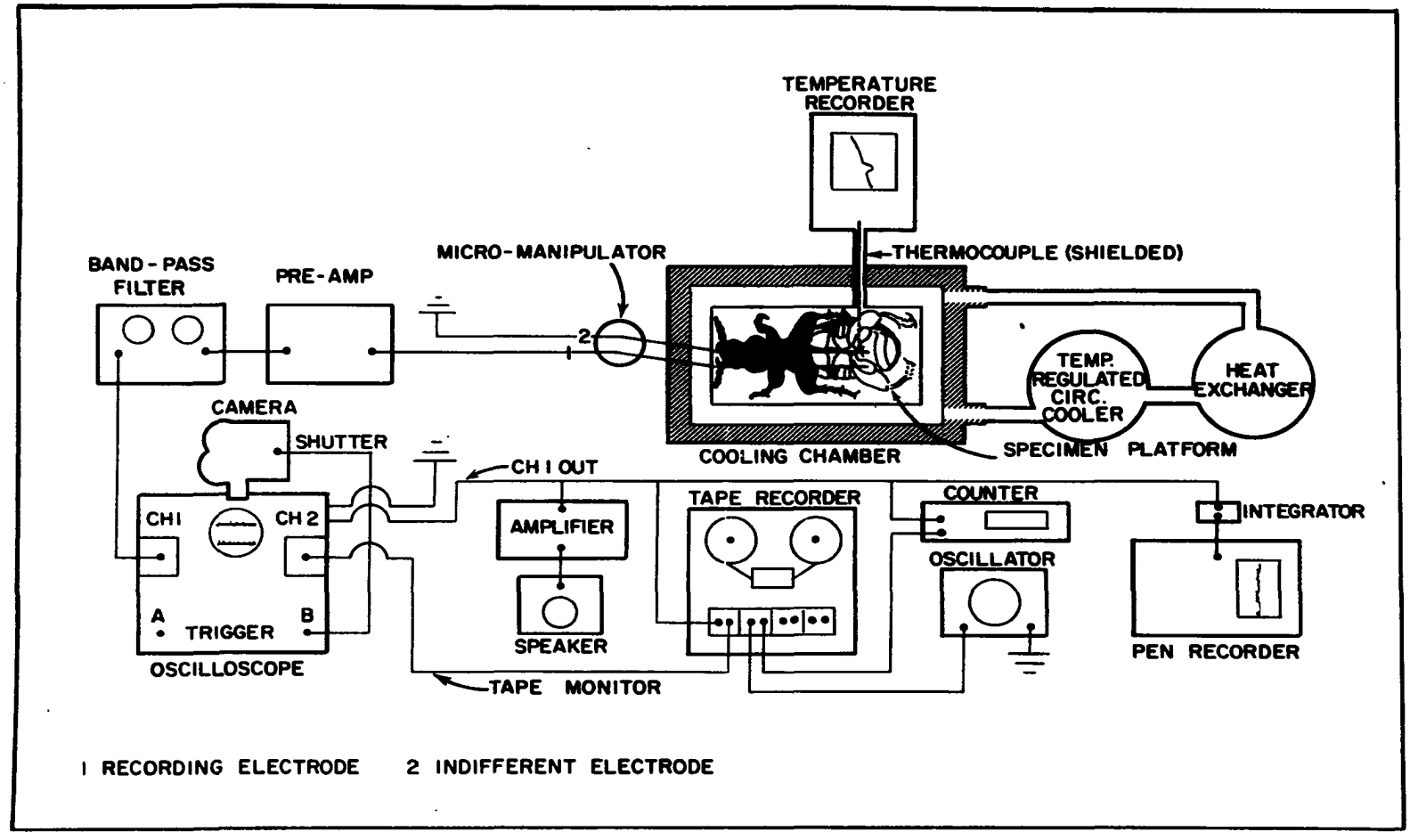


Figure 4-2. Illustrates the apparatus set up for recording from the trochanter efferents under controlled thermal conditions along with a generalized wiring schematic.

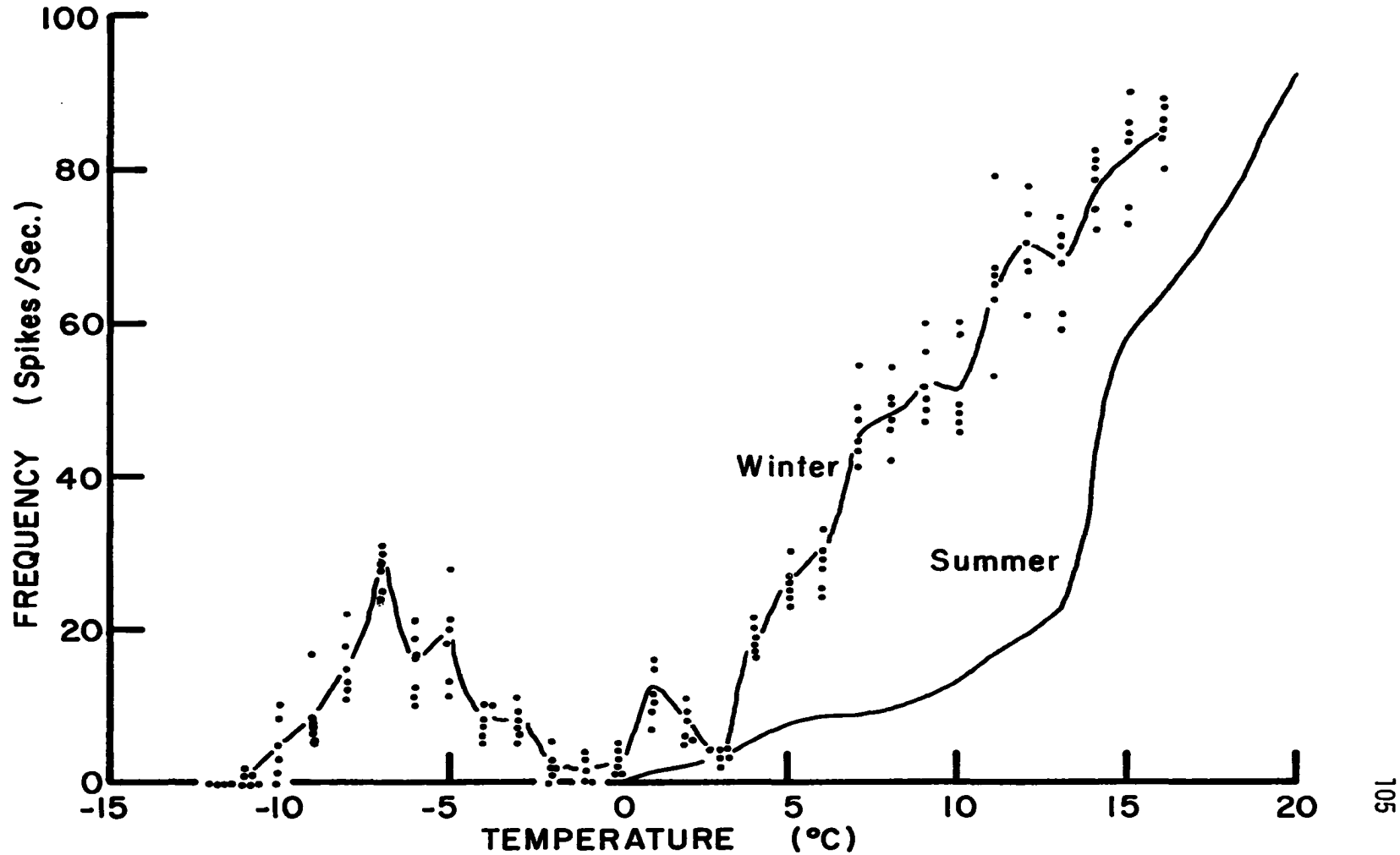


Figure 4-3. Dot frequency distribution of motor fiber discharge vs. temperature in both summer and winter beetles (P. brevicornis). Curves are mean frequency. Six sample experiments are represented for winter specimens.

of two possible motor fiber populations active over relatively broad temperature ranges (0 to 15°C and 15°C to < 20°C). This pattern is similar to those obtained by other workers' from both invertebrates and vertebrates. Winter frequency patterns, however, are unique in that they demonstrate a "peaking" phenomenon. Decreasing temperatures initially decrease the tonic discharge over a broad temperature range (< 20°C to 2°C). At this lower point, spike frequencies are observed to oscillate first upward and then downward over narrower temperature ranges until a lower thermal limit is reached, at which time all indications of neural activity terminate. Apparently, in winter beetles fiber populations are functional at narrower and more varying temperature ranges than in summer (Table 4-1).

If rewarming is attempted prior to freezing, the discharge pattern observed is nearly identical but reversed from the curve obtained during cooling in both winter and summer specimens. However, if freezing occurs in the winter condition, a hysteresis is observed. Neural activity does not appear to return until the specimen is warmed 5 to 10°C above the true freezing point (-4 to -5°C). This phenomenon is similar to that observed following rewarming after cessation of activity in mammalian nerves (Miller, 1965). Freezing in the summer condition is lethal.

Preliminary acclimation studies were conducted in an attempt to explain the mechanisms allowing for the above variations in sensitivity. Winter beetles were collected at an ambient temperature of -20°C and warmed stepwise over five-day periods. Variations in discharge patterns are represented in Table 4-2. Both the cold acclimatized and acclimated

TABLE 4-1

Thermal range of activity in the trochanter motor fibers of *P. brevicornis*.

| WINTER | | SUMMER | |
|---|---|---|---|
| Fiber Population # | Range (°C) | Fiber Population # | Range (°C) |
| 1 | <16 - 12 | 1 | <29 - 15 |
| 2 | 12 - 9 | 2 | 15 - 0 |
| 3 | 9 - 2 | | |
| 4 | 2 - (-1) | | |
| | (-1) - (-6) | | |
| 6 | (-6) - (-11) | | |

TABLE 4-2

Extinction temperatures and thermal ranges of activity of motor fibers acclimated P. brevicornis

| Acclimation State | Extinction Temp (°C) | Activity Ranges (°C) 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| "Winter" -20°C | -11.7 | <20 to 13 | 13 to 10 | 10 to 3 | 3 to -1 | -1 to -6 | -6 to -11 |
| 0°C for 5 days | -8.0 | <20 to 6 | 6 to -1 | -1 to -4 | -4 to -8 | -- | -- |
| 20°C for 10 days | -8.0 | <20 to 11 | 11 to 2 | 2 to -1 | -1 to -8 | -- | -- |
| 20°C for 11 days | -7.0 | <20 to 15 | 15 to 10 | 10 to 6 | 6 to -7 | -- | -- |
| 20°C for 14 days | 0 | <20 to 15 | 15 to 0 | -- | -- | -- | -- |

beetles show the typical winter pattern with approximately 6 fiber groups and conduction terminating at ∿ -11°C. After warming to 0°C for five days, the pattern changed. Activity ceased at -8°C and only four population ranges were evident. Following five days of warming to +20°C, the pattern continued to change. Four populations were still evident but their range of sensitivity had increased and shifted toward warmer temperatures. This adaptative trend continued until acclimation to near summer temperature was evident. With the duration of exposure to +20°C continued for eleven days, the pattern changed further. The peak amplitudes diminished greatly while the range of differential thermal sensitivity shifted further toward higher temperatures. Cessation of neural activity occurred at -7°C. Further chronic exposure to +20°C again decreased sensitivity while causing extinction temperatures to increase to 0°C. It should be noted that spike duration, which is indicative of changes in sodium and potassium permeability and therefore conduction velocity, was relatively independent of temperature in both summer and winter beetles until temperatures below 0°C were reached.

An extensive discussion of spike characteristics has not been attempted primarily due to the nature of the recording system and paucity of conclusive data. Semi-microelectrode recordings yield multifiber preparations. Identification of individual fiber spikes was not always possible in light of the changes in discharge frequency with temperature. A number of preparations were obtained in which only one to three fibers were evident. Measurements of spike durations were unusually constant over broad ranges of temperature both in summer and

winter beetles. Between 0°C and 25°C durations ranged from 1 to 2.5 msec for different fibers. Below 0°C and in cold acclimated forms, durations varied considerably. The time course of the nerve active state increased rapidly in particular fibers while others were less dependent on decreasing temperature. Durations of from 2 to 15 msec were common.

The spike durations further indicate the neural as opposed to muscle origin of the activity. There is also an indication of a broad temperature insensitivity of the factors responsible for impulse generation and propagation within the efferent fibers.

Ablation and Neurohumoral Experiments

Ablation experiments were undertaken in an attempt to demonstrate that the locus of origin of the spike activity was the motor trunk leading from the last thoracic ganglion. Nerve cord transection posterior to the last thoracic ganglion had no noticeable effect upon the activity. Transection anterior to the last thoracic ganglion resulted in only a slight observable change. Most extraneous activity was lost but the temperature dependency of discharge was not affected. Complete removal of other appendages (legs, antennae, etc.) or cutting the last thoracic leg distal to the trochanter did not influence the neural activity. Section of the connectives proximal to the trochanter and distal to the ganglion resulted in the complete termination of activity.

It is apparent from this series of experiments that the origin of the temperature dependent discharge is localized within one or a com-

bination of three places. The last thoracic ganglion is primarily implicated. The possibility exists that activity is dependent upon thermoreceptors either within the trochanter or thorax. Preliminary recordings from other portions of the same leg and even other legs reveals a distinct similarity in temperature influenced discharge patterns. This latter point would tend to confirm the first hypothesis: i.e., that the origin of activity is wholely within the last thoracic ganglion.

Various neurohumoral substances were applied to the muscle regions surrounding the recording electrode to determine what effect, if any, such substances would have on discharge frequencies. While the exact nature of neuromuscular transmitters and inhibitors is as yet unknown in many insects (Bullock and Horridge, 1965), it is of value to consider a number of basic classes of compounds known to affect conduction at this site. Since observational, surgical and spike characteristic data indicate that the observed activity is representative of nerve potentials as opposed to muscle potentials, it was felt that addition of these compounds would tend to substantiate or negate this conclusion. The following compounds representing most groups of neurohomoral compounds were individually applied without any observable effects upon recorded spikes: acetylcholine, 4-aminobutyric acid (GABA), 3-(-2-aminoethyl) indol (tryptamine), histamine, 5-hydroxytryptamine, physostigmine, procaine and curare. If the recordings were of muscle potentials, one or more of these compounds would be expected to alter the discharge in either an excitatory or inhibitory manner.

The concentrations of all the above compounds were supraphysiological.

Approximately 0.25 ul of an aqueous solution containing $10^{-3}$g/ml was applied to the recording site per injection.

## DISCUSSION

With the realization that behavioral or limited metabolic thermoregulation would be impracticable in northern latitudes where warm temperatures necessary for sustaining activities (feeding, development, etc.) are unavailable for most of the year, a mechanism allowing for continued activity at lower temperatures appears necessary. Pterostichus brevicornis appears to have developed a mechanism by which the nervous system can become adapted so as to remain functional at very low temperatures.

Electrical recordings from the motor fibers of the trochanter muscles of the hind legs demonstrate in winter beetles differential temperature sensitivities. Various groups of fibers were active over different temperature ranges due to variable cold blocking.

The summer beetle, however, demonstrates a different but typical phenomenon. Spike frequency patterns are similar to those previously recorded in cold stressed poikilotherms. Efferent discharge frequencies decrease with decreasing temperature in an exponential fashion with only two populations apparent.

When winter beetles are gradually and artificially warmed, the fiber populations are observed to decrease in number while shifting their range of activity to warmer temperatures. During this same period, extinction points for neural activity are observed to increase.

This gradual change in sensitivity explains the distinct differences between summer and winter forms.

The value of such a mechanism is easily understood when one considers that great variations in ambient temperature are experienced by this insect. In spring and fall temperatures can change more than 20°C within minutes and greater overall variations can occur in winter. Such acute temperature drops inactivate less hardy insects and would certainly kill most. This beetle, however, continues to be active (within limits).

P. brevicornis differs from many other northern insects in that it has a relatively long development period and life cycle. Herein lies the value of its ability to extend activity period during early frost and to continue activity during winter on occasional warm days.

One can only speculate as to the mode by which this mechanism operates. The peaks of activity at low temperatures apparently reflect recruitment of additional motor units that had been inactive at higher temperatures. This, however, implies changed motor function, i.e. local thermogenesis, which might seem improbable. Increased tone in various units is thought to be a more plausible conclusion. Cooling rates do not change the pattern (i.e. 0.5°C/min to 4°C/min), nor does frequency change when the insect is held at a constant temperature for periods as long as three hours. These observations tend to discount the questions of thermal gradients acting on higher nervous centers, of electrode polarization artifacts or of struggling.

## SUMMARY

1. P. brevicornis has been found to overwinter in the adult stage within a decayed stump hibernaculum. The stump offers a suitable habitat by a) buffering fluctuations in air temperature, b) reducing cooling rates and c) re-warming rapidly.
2. Seasonal variations in temperature preferences have been demonstrated. Specimens avoided sub-freezing conditions unless glycerol accumulated within the hemolymph.
3. Coordinated neuromuscular activities (walking, climbing, etc.) were evident at temperatures as low as -12°C in the cold hardened specimens.
4. Hemolymph levels of glycerol and trehalose were found to vary seasonally. Hemolymph glucose levels were constant.
5. Variations in whole body supercooling and hemolymph freezing points were found to directly correlate with changes in the hemolymph glycerol concentrations.
6. The initial stimulus to glycerol accumulation (synthesis) was found to be exposure to 0°C following summer in both the naturally acclimatized and laboratory acclimated specimens.
7. Variations in ambient temperature were reflected in the changes in hemolymph glycerol concentrations at temperatures as low as -50°C in outdoor specimens. Changes in the concentration of this same substance varied with temperature exposure in the laboratory.
8. Acute exposure to above freezing temperatures (0°C) resulted

in the rapid loss of glycerol and elevation of supercooling and freezing points.

9. Glycerol is considered to be the major cryoprotective compound (solute) present in this insect. Its theoretical mechanisms of protection have been discussed. No other cryoprotective substance has been detected.
10. Various physico-chemical aspects of the effects of glycerol on aqueous solutions and ice crystal structure have been considered. The effects of glycerol on these factors may be interpreted as protective to tissues upon extracellular freezing.
11. Neuromuscular function was studied and found to vary in response to temperature in both the acclimatized and acclimated specimens thus indicating a temperature induced neural adaptation.
12. Patterns of motor fiber discharge varied seasonally and during laboratory acclimation. Neural extinction temperatures were lowered in the cold hardened form while elevated in the warm adapted specimens.
13. Cold hardened forms demonstrated narrower ranges of neural activity in response to temperature changes. These changes are thought to reflect changes in nerve tone rather than changes in recruitment.
14. Pterostichus brevicornis has been shown to adapt behaviorally, biochemically and neuromuscularily to low temperature in a manner demonstrating varied and potentiated survival advantages to life in the sub-arctic.

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